

increased capillary density and organized capillary network in the engineered myocardial tissues, enhanced GFP-labeled EPCs originating from the transplanted cell sheet seemed to differentiate into an inner vWF- and vascular endothelial-cadherin-positive endothelial layer surrounded by an outer circumferential SMA-positive layer, partially derived from transplanted SMCs. The direct contribution of SMCs was confirmed by fluorescence in situ hybridization analysis of the myocardium, demonstrating new vasculature containing male SMCs in a female heart. Furthermore, the morphology of the vessel formation within myocardial tissues, including the diameter, composition, and stability of vessel walls, suggested that vessel maturation may occur under pathological stimuli. Furthermore, our data showed that coculturing EPCs with SMCs enhanced the secretion of TGF β , which is thought to promote stabilization in multiple ways: the synthesis and deposition of ECM and contextual regulation of proliferation and differentiation.¹⁷ Therefore, it is likely that the process of vessel maturation is a transition from an actively growing vessel to a quiescent fully functional mature vessel network via endothelial-pericyte interaction.

The mechanism by which the transplanted cocultured bi-level cell sheet attenuated ventricular remodeling and improved cardiac function, as shown in this study, seemed to depend on the cell sheet being placed over the scarred area of the myocardium and led to repair of the anterior wall thickness, reduction of LV wall stress, and the improvement of LV function. Previous studies indicated that the surviving myocardium and transplanted cell sheet attenuate complex cellular and molecular events, including hypertrophy, fibrosis, apoptosis of the myocardium, and the pathological accumulation of ECM.^{7,23}

Cell engraftment is another critical aspect of myocardial regeneration. The potential advantages of the cell-sheet technology include the ability to deliver a larger number of transplanted cells that integrate with native tissues without destroying the cell-cell or cell-ECM adhesions in the cell-sheet.⁷ Together with our significant findings of increased cell survival, integrin β 1 upregulation, and the enhanced secretion of HGF in vitro in the cell-sheet group, it is likely that the cocultured bi-level cell-sheet prolonged cell survival by preventing anoikis mediated by the ECM receptors, in particular via integrin β 1, or modulated by growth factor (eg, HGF).²⁴

This treatment strategy for acute myocardial infarction is not yet directly applicable to the clinical arena because of the time required to isolate, cultivate, and manipulate cells in vitro. However, the finding that this therapy yielded marked cardioprotective effects through angiogenesis should be beneficial for treating other types of cardiac pathologies, such as the chronic phase of myocardial infarction.

A potential limitation of this study is that the optimal number of transplanted cells was unknown in vivo. In addition, further studies are necessary to determine the optimal mixing ratio of transplanted EPCs and SMCs. We believe that this scaffold-free cell-sheet technique seems to be more transplantable to humans.¹⁵ Although the cocultured bi-level cell sheet maintained different cell types in separate layers in vitro, our in vivo findings showed that the transplanted cell sheet could be a mixture of both cell types. This is probably because each

cell type possessed different cell affinity, cell-matrix attachment, and migration ability.

In conclusion, we found that coculturing EPCs with SMCs in a bi-level cell-sheet delivery system enhanced the angiogenic effect by facilitating more architecturally mature microvascular formation. We also observed that bi-level cell-sheet technology initiated robust angiogenesis and regulated vessel maturation, thereby reducing fibrosis, attenuating ventricular remodeling, and improving cardiac function in ischemic cardiomyopathic rats. These findings suggest that novel bi-level cell-sheet technology creates an avenue of powerful cardiac repair. This concept may lead to new regeneration therapies in advanced cardiomyopathy.

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Disclosures

None.

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Evaluation of intramitochondrial ATP levels identifies G0/G1 switch gene 2 as a positive regulator of oxidative phosphorylation

Hidetaka Kioka^{a,b,1}, Hisakazu Kato^{a,1}, Makoto Fujikawa^c, Osamu Tsukamoto^a, Toshiharu Suzuki^{d,e}, Hiromi Imamura^f, Atsushi Nakano^{a,g}, Shuichiro Higo^{a,b}, Satoru Yamazaki^h, Takashi Matsuzaki^b, Kazuaki Takafujiⁱ, Hiroshi Asanuma^j, Masanori Asakura^g, Tetsuo Minamino^b, Yasunori Shintani^a, Masasuke Yoshida^e, Hiroyuki Noji^k, Masafumi Kitakaze^g, Issei Komuro^{b,1}, Yoshihiro Asano^{a,b,2}, and Seiji Takashima^{a,2}

Departments of ^aMedical Biochemistry and ^bCardiovascular Medicine and ¹Center for Research Education, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan; ²Department of Biochemistry, Faculty of Pharmaceutical Science, Tokyo University of Science, Chiba 278-8510, Japan; ³Chemical Resources Laboratory, Tokyo Institute of Technology, Yokohama 226-8503, Japan; ⁴Department of Molecular Bioscience, Kyoto Sangyo University, Kyoto 603-8555, Japan; ⁵The Hakubi Center for Advanced Research and Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan; Departments of ⁶Clinical Research and Development and ⁷Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Osaka 565-8565, Japan; ⁸Department of Cardiovascular Science and Technology, Kyoto Prefectural University School of Medicine, Kyoto 602-8566, Japan; and ⁹Department of Applied Chemistry, School of Engineering and ¹⁰Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-8656, Japan

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The oxidative phosphorylation (OXPHOS) system generates most of the ATP in respiring cells. ATP-depleting conditions, such as hypoxia, trigger responses that promote ATP production. However, how OXPHOS is regulated during hypoxia has yet to be elucidated. In this study, selective measurement of intramitochondrial ATP levels identified the hypoxia-inducible protein G0/G1 switch gene 2 (G0s2) as a positive regulator of OXPHOS. A mitochondria-targeted, FRET-based ATP biosensor enabled us to assess OXPHOS activity in living cells. Mitochondria-targeted, FRET-based ATP biosensor and ATP production assay in a semi-intact cell system revealed that G0s2 increases mitochondrial ATP production. The expression of G0s2 was rapidly and transiently induced by hypoxic stimuli, and G0s2 interacts with OXPHOS complex V (F_0F_1 -ATP synthase). Furthermore, physiological enhancement of G0s2 expression prevented cells from ATP depletion and induced a cellular tolerance for hypoxic stress. These results show that G0s2 positively regulates OXPHOS activity by interacting with F_0F_1 -ATP synthase, which causes an increase in ATP production in response to hypoxic stress and protects cells from a critical energy crisis. These findings contribute to the understanding of a unique stress response to energy depletion. Additionally, this study shows the importance of assessing intramitochondrial ATP levels to evaluate OXPHOS activity in living cells.

energy metabolism | live-cell imaging

Maintaining cellular homeostasis and activities requires a stable energy supply. Most eukaryotic cells generate ATP as their energy currency mainly through the mitochondrial oxidative phosphorylation (OXPHOS) system. The OXPHOS system consists of five large protein complex units (i.e., complexes I–V), comprising more than 100 proteins. In this system, oxygen (O_2) is essential as the terminal electron acceptor for complex IV to finally produce the proton-motive force that drives the ATP-generating molecular motor complex V (F_0F_1 -ATP synthase).

Hypoxia causes the depletion of intracellular ATP and triggers adaptive cellular responses to help maintain intracellular ATP levels and minimize any deleterious effects of energy depletion. Although the metabolic switch from mitochondrial respiration to anaerobic glycolysis is widely recognized (1–4), several recent reports have shown that hypoxic stimuli unexpectedly increase OXPHOS efficiency as well (5–7). In other words, cells have adaptive mechanisms to maintain intracellular ATP levels by enhancing OXPHOS, particularly in the early phase of hypoxia, in which the O_2 supply is limited but still remains. However, the mechanism by which OXPHOS is regulated during this early hypoxic phase is still not fully understood.

Revealing the mechanism of this fine-tuned regulation of OXPHOS requires accurate and noninvasive measurements of OXPHOS activity. Although researchers have established methods to measure OXPHOS activity, precise measurement, especially in living cells, is still difficult. Measuring the intracellular ATP concentration is one of the most commonly used methods for evaluating OXPHOS activity. However, there are two major problems with this method. First, the intracellular ATP concentration does not always accurately reflect OXPHOS activity, because it can also be affected by glycolytic ATP production, cytosolic ATPases, and ATP buffering enzymes, such as creatine kinase and adenylate kinase (8). Second, because measurements of the ATP concentration by chromatography (9), MS (10), NMR (11), or luciferase assays (12) are based on cell extract analysis, these methods cannot be used to measure the serial ATP concentration changes in living cells in real time.

In this study, we overcame these problems by the selective measurement of the intramitochondrial matrix ATP concentration ($[ATP]_{mito}$) in living cells. In the final step of OXPHOS, ATP is produced not in the cytosol but in the mitochondrial matrix. Therefore, we hypothesized that a selectively measuring $[ATP]_{mito}$ is suitable for the highly sensitive evaluation of cellular ATP production by OXPHOS. In fact, real-time evaluation of both $[ATP]_{mito}$ and the cytosolic ATP concentration ($[ATP]_{cyto}$) in living cells revealed that $[ATP]_{mito}$ reflected OXPHOS activity with far more sensitivity than $[ATP]_{cyto}$. Using this fine method, we found that G0/G1 switch gene 2 (G0s2), a hypoxia-induced

Significance

We developed a sensitive method to assess the activity of oxidative phosphorylation in living cells using a FRET-based ATP biosensor. We then revealed that G0/G1 switch gene 2, a protein rapidly induced by hypoxia, increases mitochondrial ATP production by interacting with F_0F_1 -ATP synthase and protects cells from a critical energy crisis.

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¹H. Kioka and H. Kato contributed equally to this work.

²To whom correspondence may be addressed. E-mail: asano@cardiology.med.osaka-u.ac.jp or takasima@cardiology.med.osaka-u.ac.jp.

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protein in cardiomyocytes, increases OXPHOS activity. G0s2 interacted with F_0F_1 -ATP synthase and increased the ATP production rate. Our results suggest that hypoxia-induced protein G0s2 is a positive regulator of OXPHOS and protects cells by preserving ATP production, even under hypoxic conditions.

Results

Establishment of a Sensitive Method to Assess OXPHOS Activity in Living Cells. To elucidate the mechanism by which OXPHOS is regulated under hypoxia, it is essential to establish a sensitive method for assessing OXPHOS activity in living cells. For this purpose, we used an ATP indicator based on ϵ -subunit for analytical measurements (ATeam), which is an ATP-sensing FRET-based indicator (13). We introduced this ATP biosensor into cardiomyocytes that possess an abundance of mitochondria and produce the highest levels of ATP among all primary cells (14, 15). The ATeam assay can measure both $[ATP]_{cyto}$ (i.e., the Cyto-ATeam assay) and $[ATP]_{mito}$ when a duplex of the mitochondrial targeting signal of cytochrome *c* oxidase subunit VIII is attached to the indicator (i.e., the Mit-ATeam assay). In this case, the YFP/CFP emission ratio of the ATeam fluorescence represents the ATP concentration in each compartment. Interestingly, the Mit-ATeam assay was a far more sensitive method than the Cyto-ATeam assay in determining OXPHOS activity in living cells. For example, a very low dose of oligomycin A (0.01 μ g/mL), a specific OXPHOS complex V (F_0F_1 -ATP synthase) inhibitor, greatly reduced the YFP/CFP emission ratio of the Mit-ATeam fluorescence that represents $[ATP]_{mito}$ within 10 min (Fig. 1*A*, *Upper* and *B* and Movie S1). In contrast, the same dose of oligomycin A resulted in a slight and slow decline of the YFP/CFP emission ratio of Cyto-ATeam fluorescence (Fig. 1*A*, *Lower* and *B* and Movie S1). The same phenomenon was observed when the cells were exposed to hypoxia, which suppresses the activity of OXPHOS complex IV (cytochrome *c* oxidase). Again, $[ATP]_{mito}$ decreased more markedly than $[ATP]_{cyto}$ during 2.5 h of hypoxia (Fig. 1*C* and *D* and Movie S2). These results indicate that the Mit-ATeam assay is far more sensitive for measuring the activity of OXPHOS than the Cyto-ATeam

assay. In addition, OXPHOS inhibition decreased the YFP/CFP emission ratio of the Mit-ATeam fluorescence of HeLa cells as well as cardiomyocytes (Fig. S1), suggesting the broad applicability of this assay. Therefore, we used Mit-ATeam for the assessment of the OXPHOS activity in living cells.

Hypoxia-Induced Gene G0s2 Affects the Intramitochondrial ATP Concentration. The expression of genes involved in OXPHOS regulation is considered to be up-regulated in the early phase of hypoxia. Thus, to find unique OXPHOS regulators, we focused on the rapidly induced genes in response to hypoxic stimulation. We compared the gene expression profiles of cultured rat cardiomyocytes at three different time points during hypoxic conditions (0, 2, and 12 h) (Fig. S2*A*). The expression of well-known hypoxia-induced genes, such as VEGF- α and hexokinase 2 mRNA (16, 17), was slightly up-regulated at 2 h and further enhanced at 12 h of hypoxia. In contrast, three other genes (*Adams1*, *Cdkn3*, and *G0s2*) underwent rapid increases in expression at 2 h but declined at 12 h of sustained hypoxia (Fig. S2*B* and *C*). This rapid and transient time course of expression implies that these three genes may play distinct regulatory roles, especially in the early hypoxic phase, in which oxygen is limited but still available. To examine whether these genes are involved in the regulation of OXPHOS activity, we knocked down these genes by shRNA (see Fig. S7*A*) and examined $[ATP]_{mito}$ using the Mit-ATeam assay. In this experiment, $[ATP]_{mito}$ in cardiomyocytes treated with shRNA for G0s2 clearly declined within 24 h compared with the control cardiomyocytes (Fig. 2*A* and Movie S3). In addition, the time course of ATP decline was in agreement with the time course of G0s2 depletion (Fig. 2*A* and Fig. S3*A*). Importantly, the over-expression of G0s2 restored normal ATP levels (Fig. 2*B* and *C*), and again, the Cyto-ATeam assay could not detect a significant effect of G0s2 knockdown within this time frame (Fig. S3*B* and Movie S4). These findings imply that mitochondrial ATP production through OXPHOS was inhibited by G0s2 ablation. We confirmed that the mRNA and protein levels of G0s2 both increased after 2–6 h of hypoxia and then declined after 12 h of hypoxia (Fig. 2*D* and *E*). G0s2 was first reported as a gene with

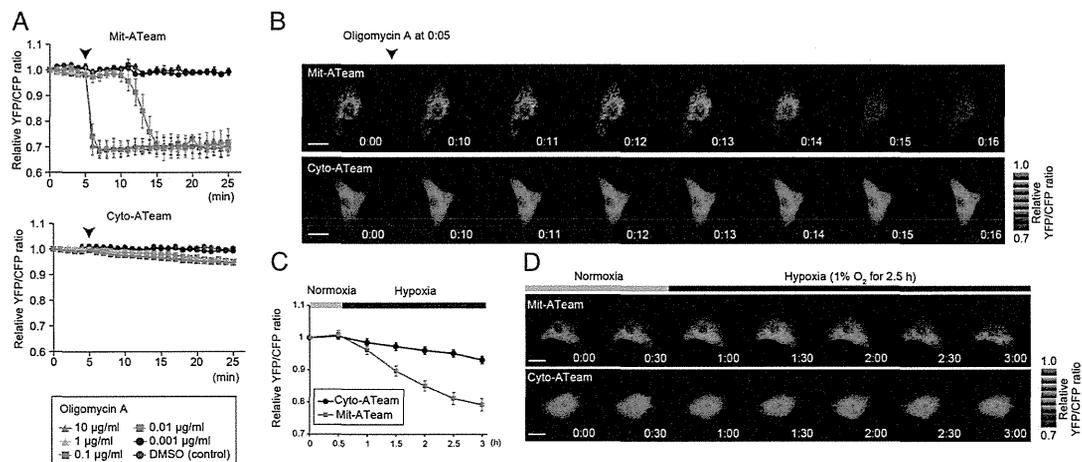


Fig. 1. Establishment of a sensitive method to assess OXPHOS activity in living cells. (*A*) YFP/CFP emission ratio plots of (*Upper*) Mit-ATeam and (*Lower*) Cyto-ATeam fluorescence in cardiomyocytes. Various concentrations (0.001, 0.01, 0.1, 1, and 10 μ g/mL) of oligomycin A or DMSO (control) were added at 5 min (arrowhead; $n = 3$). (*B*) Representative sequential YFP/CFP ratiometric pseudocolored images of (*Upper*) Mit-ATeam and (*Lower*) Cyto-ATeam in cardiomyocytes. Oligomycin A (0.01 μ g/mL) was added at 5 min. (Scale bars: 20 μ m.) (*C*) YFP/CFP emission ratio plots of Mit-ATeam and Cyto-ATeam fluorescence in cardiomyocytes ($n = 10$). (*D*) Representative sequential YFP/CFP ratiometric pseudocolored images of (*Upper*) Mit-ATeam and (*Lower*) Cyto-ATeam in cardiomyocytes. Cells were exposed to 1% hypoxia from the time point 30 min. All of the measurements were normalized to the YFP/CFP emission ratio at 0 min. Data are represented as the means \pm SEMs. (Scale bars: 20 μ m.)

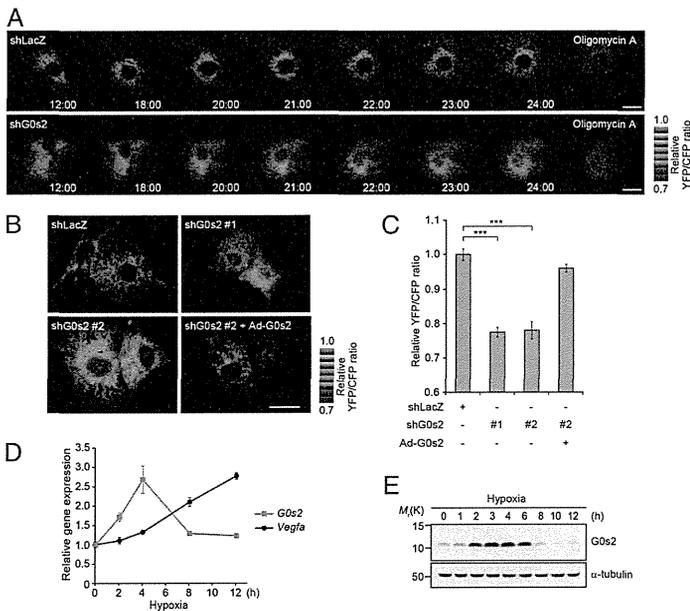


Fig. 2. G0s2, a hypoxia-inducible protein, affects intramitochondrial ATP concentration in cardiomyocytes. (A) Sequential YFP/CFP ratiometric pseudocolored images of Mit-ATeam fluorescence in cardiomyocytes expressing (Upper) shRNAs for LacZ (shLacZ) or (Lower) G0s2 (shG0s2). Oligomycin A (1 μ g/ml) was added at the end of the time-lapse imaging to completely inhibit ATP synthesis. The indicated time represents the period after adenovirus infection. (B) Representative YFP/CFP ratiometric pseudocolored images of Mit-ATeam fluorescence in cardiomyocytes expressing the indicated adenovirus for 24 h. (Scale bar: A and B, 20 μ m.) (C) The bar graph shows the mean YFP/CFP emission ratio of Mit-ATeam fluorescence in cardiomyocytes expressing shLacZ ($n = 30$), shG0s2 #1 ($n = 30$), shG0s2 #2 ($n = 29$), and shG0s2 #2 + G0s2 WT ($n = 32$) for 24 h. All of the measurements were normalized to the average of the control cells (shLacZ). *** $P < 0.001$. (D) Gene expression value plots of G0s2 (red line) and VEGF- α (Vegfa; black line) levels in cardiomyocytes under hypoxic conditions (1% O₂). Each value was compared with the level of Actb expression ($n = 3$). Values represent the means \pm SEMs. (E) Immunoblotting of the G0s2 expression in cardiomyocytes under hypoxic conditions (1% O₂).

expression that was induced during the cell cycle switch from G0 to G1 phase (18). G0s2 is expressed in many tissues and especially abundant in heart, skeletal muscle, liver, kidney, brain, and adipose tissue (19). Although G0s2 may play a role in cell cycle progression (20), the function of G0s2 in the hypoxic response remains unknown.

G0s2 Rescues the Decline of ATP Production During Hypoxia. We next tested whether the overexpression of the G0s2 before hypoxic stress could prevent hypoxia-induced ATP depletion. We prepared cardiomyocytes overexpressing G0s2 and control cardiomyocytes. During sustained hypoxia, [ATP]_{mito} gradually declined in control cardiomyocytes as measured by the Mit-ATeam assay. Notably, the overexpression of G0s2 before the onset of hypoxia reduced this decline in [ATP]_{mito}, which allowed the cardiomyocytes to promptly recover to baseline levels of [ATP]_{mito} after reoxygenation (Fig. 3A and B and Movie S5). In addition, the prehypoxia overexpression of G0s2 preserved cell viability during sustained hypoxia (Fig. 3C). These results suggest that G0s2 can preserve

mitochondrial ATP production even under hypoxia and protect cells from the energy crisis under hypoxia.

G0s2 Binds to F₀F₁-ATP Synthase but Not Other OXPHOS Protein Complexes. To reveal the mechanism by which G0s2 affects [ATP]_{mito}, we sought to identify the biochemical targets of G0s2. We screened for G0s2 binding proteins by immunoprecipitation of cell lysates from cardiomyocytes expressing C-terminally Flag-tagged G0s2 (G0s2-Flag). G0s2-Flag is expressed in cardiomyocytes localized to the mitochondria (Fig. S4A). MS analysis revealed that multiple F₀F₁-ATP synthase subunits, but no other mitochondrial respiratory chain complex subunits, were coimmunoprecipitated with G0s2-Flag (Fig. S4B and Table S1). F₀F₁-ATP synthase is a well-known ATP-producing enzyme composed of a protein complex that contains an extramembranous F₁ and an intramembranous F₀ domain linked by a peripheral and a central stalk (21–24). The binding of F₀F₁-ATP synthase to G0s2-Flag was confirmed by immunoblotting with antibodies against several subunits of F₀F₁-ATP synthase (Fig. 4A).

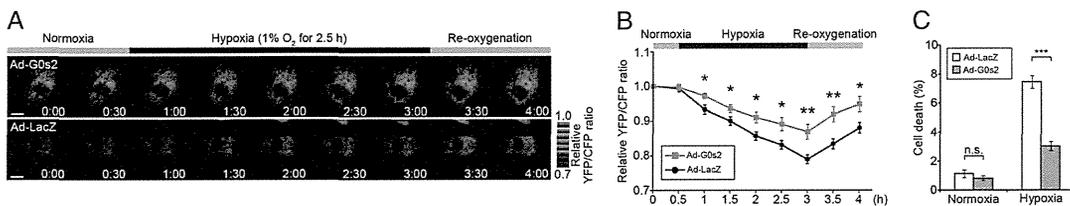


Fig. 3. Overexpression of G0s2 before hypoxia rescues the decline of mitochondrial ATP production during hypoxia. (A) Sequential YFP/CFP ratiometric pseudocolored images of Mit-ATeam fluorescence in cardiomyocytes expressing (Upper) G0s2 WT or (Lower) LacZ during hypoxia and reoxygenation. (Scale bar: 20 μ m.) (B) YFP/CFP emission ratio plots of Mit-ATeam fluorescence in cardiomyocytes expressing G0s2 WT ($n = 20$) or LacZ ($n = 19$) during hypoxia and reoxygenation. All of the measurements were normalized to the ratio at time 0 and compared between cardiomyocytes with G0s2 WT and LacZ at each time point. (C) The bar graph shows the cell viability of cardiomyocytes overexpressing G0s2 under hypoxic conditions. Cardiomyocytes expressing either LacZ or G0s2 WT were cultured under normoxic or hypoxic conditions for 18 h ($n = 8$). The asterisks denote statistical significance comparing G0s2 with LacZ. Data are represented as the means \pm SEMs. n.s., not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Conversely, G0s2-Flag was coimmunoprecipitated with F_0F_1 -ATP synthase (Fig. S4C). G0s2-Flag was also found to be associated with the F_0F_1 -ATP synthase in 293T and HeLa cells (Fig. S4C). Both coimmunoprecipitation using an anti-G0s2 antibody and a reciprocal immunoprecipitation revealed that endogenous G0s2 interacts with F_0F_1 -ATP synthase, whereas none of the proteins in complexes I–IV or adenine nucleotide translocase 1 (ANT1; also referred to as ADP/ATP carrier) were coimmunoprecipitated with G0s2 (Fig. 4B and C).

Given that the G0s2 protein contains an evolutionarily conserved amino terminus and one hydrophobic domain (HD) (19), we created three G0s2 partial deletion mutants to identify the domain in G0s2 that is important for binding to F_0F_1 -ATP synthase (Fig. S4D). Among these mutants, G0s2 Δ C and G0s2 Δ N but not G0s2 Δ HD bound to the F_0F_1 -ATP synthase complex (Fig. 4D and Fig. S4E and F). Furthermore, we confirmed that G0s2 directly interacts with F_0F_1 -ATP synthase in an *in vitro* pull-down assay using a recombinant maltose-binding protein–fused G0s2 protein and purified F_0F_1 -ATP synthase from bovine heart mitochondria (Fig.

S5). Immunocytochemical analysis revealed that endogenous G0s2 colocalized with the β -subunit of F_0F_1 -ATP synthase (Fig. 4E). The knockdown of G0s2 expression by shRNA abolished G0s2 staining (Figs. S6 and S7A), indicating that both antibodies used for immunostaining specifically recognize G0s2. These data suggest that G0s2 interacts with the F_0F_1 -ATP synthase complex through its HD in mitochondria and regulates OXPHOS activity.

G0s2 Increases Mitochondrial ATP Production Rate. $[ATP]_{mito}$ is mainly determined by the rate of ATP synthesis by F_0F_1 -ATP synthase and ATP/ADP exchange by the ATP/ADP translocase ANT1. This theory means that the increased $[ATP]_{mito}$ observed in the G0s2-overexpressing cells may result from the increased ATP synthesis and/or decreased ATP/ADP exchange, although G0s2 did not interact with ANT1 (Fig. 4B). To resolve this issue and directly measure the rate of ATP production in mitochondria, we used a semiintact cell system called the mitochondrial activity of streptolysin O permeabilized cells (MASC) assay (25). In this assay, we permeabilized the plasma membrane to wash out any cytosolic components, such as creatine and glycolytic substrates, but left the mitochondria intact. Furthermore, we treated the cells with P^1 , P^3 -di(adenosine-5') pentaphosphate to inhibit the activity of adenylate kinase. These steps allowed us to measure the ATP production rate mostly from OXPHOS, with a minimal contribution of ATP buffering systems in the cytosol. The MASC assay was suitable for accurate measurement of mitochondrial ATP production rate, because mitochondria in this semiintact cell system suffered much smaller damage than the isolated mitochondria in the conventional method. Surprisingly, in the MASC assay, the ATP production rate markedly increased when G0s2 was expressed in HeLa cells that lacked endogenous G0s2 (Fig. 5A). In cardiomyocytes, shRNA-mediated G0s2 knockdown decreased the ATP production rate in mitochondria, and the expression of G0s2 WT but not G0s2 Δ HD could restore the ATP production rate (Fig. 5B and Fig. S7A). In both cells, complete inhibition of ATP production by oligomycin A indicated that the observed ATP synthesis was catalyzed by OXPHOS but not other metabolism (Fig. 5A and B).

Next, to evaluate the physiological role of G0s2, we examined whether endogenous G0s2 induced by hypoxia could enhance the ATP production rate. Cardiomyocytes were pretreated with hypoxia for 4 h, during which G0s2 expression was largely induced. We then evaluated the ATP production rate of both hypoxia-pretreated and nontreated cardiomyocytes under room air conditions. Even under these equivalent normoxic conditions, hypoxia-pretreated cardiomyocytes produced ATP faster than nontreated control cardiomyocytes (Fig. 5C and Fig. S7B). G0s2 knockdown attenuated this increase in the rate of ATP production, indicating that the enhanced ATP production rate resulting from hypoxia pretreatment primarily depends on endogenous G0s2 induction. This increased G0s2 expression was essential for cell survival, because G0s2-depleted cells died earlier than control cells under conditions of hypoxic stress (Fig. 5D).

Furthermore, to assess the effect of G0s2 on cellular respiration, we continuously measured the oxygen consumption rate (OCR) using an XF96 Extracellular Flux Analyzer. G0s2 knockdown decreased the basal OCR of cardiomyocytes, most likely because of the decreased activity of ATP synthesis (Fig. 5E and F). In contrast, the proton leakage of the mitochondrial inner membrane and the maximum respiratory capacity of OXPHOS complexes I–IV were unaffected by G0s2 ablation (Fig. 5E and F). These data show that G0s2 knockdown reduced respiration caused by ATP synthesis without affecting respiration caused by proton leakage, nonmitochondrial respiration, or the maximal respiration capacity.

All these findings indicate that G0s2 enhances the mitochondrial ATP production rate by increasing the activity of F_0F_1 -ATP synthase.

Discussion

In this study, we showed that G0s2 kinetically increased OXPHOS activity through direct binding to F_0F_1 -ATP synthase. Our previous

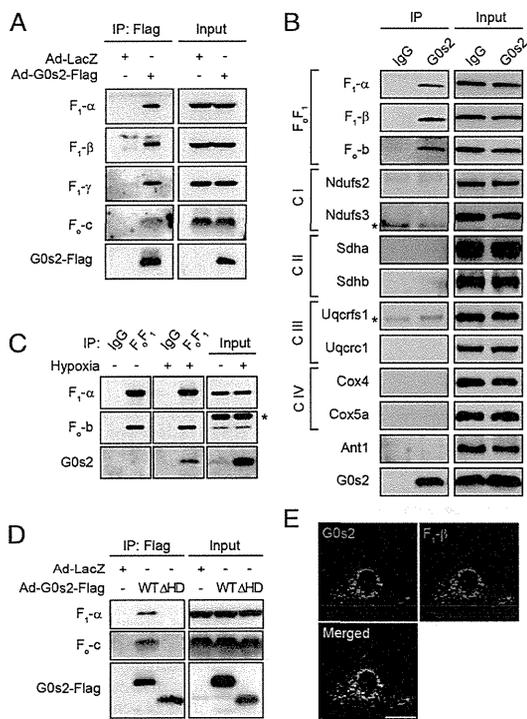


Fig. 4. G0s2 interacts with the F_0F_1 -ATP synthase in mitochondria. (A) Immunoprecipitation (IP) of G0s2-Flag in cardiomyocytes. Cell lysates from cardiomyocytes expressing G0s2-Flag or LacZ were immunoprecipitated with an anti-Flag antibody. (B) IP of endogenous G0s2 in cardiomyocytes. Endogenous G0s2 was induced by hypoxia and immunoprecipitated using an anti-G0s2 antibody. C, OXPHOS complex; F_0F_1 , F_0F_1 -ATP synthase. *IgG light chain. (C) IP of F_0F_1 -ATP synthase in cardiomyocytes under normoxic or hypoxic conditions. Cell lysates from cardiomyocytes cultured under normoxia or hypoxia for 4 h were immunoprecipitated with an antibody against the whole F_0F_1 -ATP synthase complex or a control IgG. *Nonspecific band. (D) IP of G0s2 mutants expressed in cardiomyocytes. Cell lysates were immunoprecipitated with an anti-Flag antibody. (E) Immunostained images of hypoxia-stimulated (4 h) cardiomyocytes with anti-G0s2 (green) and anti- F_0F_1 -ATP synthase β -subunit (red) antibodies. (Scale bars: 20 μ m.)

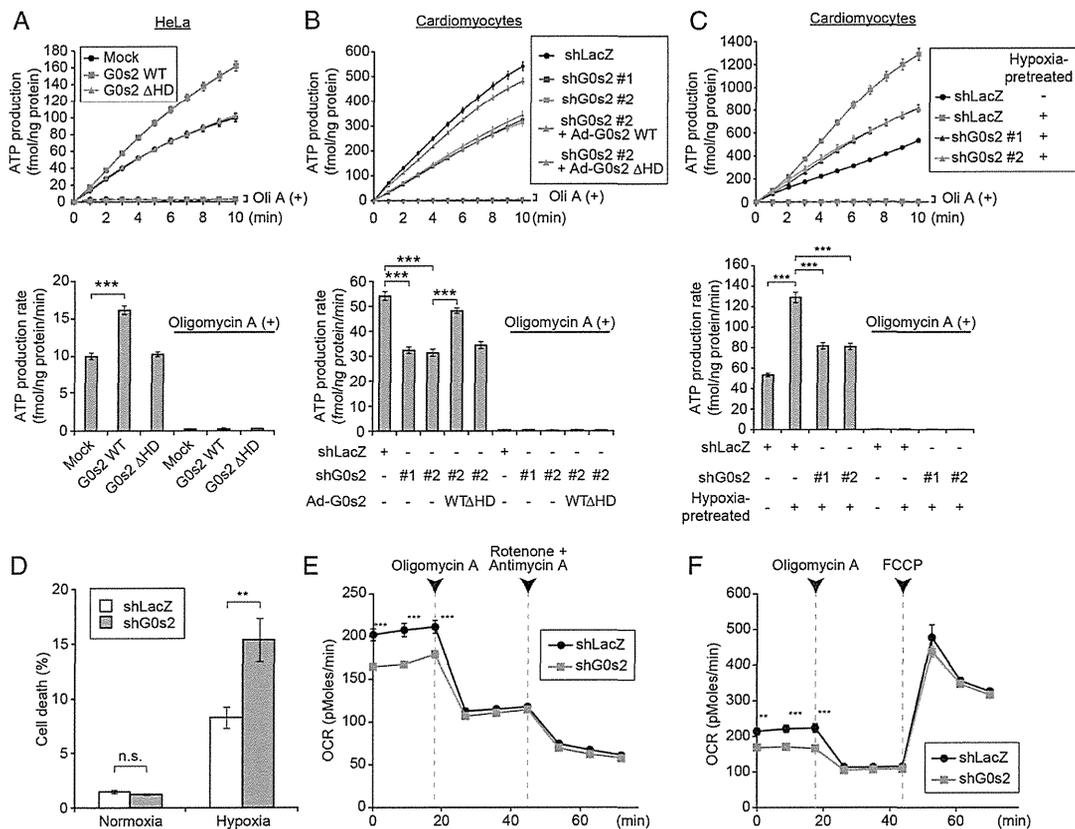


Fig. 5. G0s2 enhances the mitochondrial ATP production rate. (A and B) MASC assay of (A) permeabilized HeLa cells expressing the indicated plasmids or (B) cardiomyocytes expressing the indicated adenovirus in the presence (dotted lines) or absence (solid lines) of 1 μ M oligomycin A (Oli A). Upper shows the ATP production plots, and Lower shows the mean ATP production rates between 0 and 10 min. (A) $n = 12$. (B) Solid lines, $n = 12$; dotted lines, $n = 8$. (C) MASC assay of permeabilized cardiomyocytes pretreated with hypoxia. Cells expressing the indicated adenovirus were pretreated with or without hypoxia for 4 h. After the pretreatment, the cells were permeabilized under room air conditions followed by MASC assay in the presence (dotted lines; $n = 8$) or absence (solid lines; $n = 12$) of 1 μ M Oli A. Upper shows the ATP production plot, and Lower shows the mean ATP production rate between 0 and 10 min. (D) The bar graph represents the cell viability of G0s2-depleted cardiomyocytes under hypoxic conditions. Cardiomyocytes expressing shLacZ or shG0s2 (#2) were cultured under normoxic or hypoxic conditions for 18 h. (E and F) The OCR in cardiomyocytes expressing shLacZ and shG0s2 (#2) under basal conditions and in response to the indicated mitochondrial inhibitors ($n = 8$). FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone. Data are represented as the means \pm SEMs. n.s., not significant. ** $P < 0.01$; *** $P < 0.001$.

studies of F_0F_1 -ATP synthase have revealed that this enzyme has a specific structure that connects two molecular nanomotors that synchronize with each other to produce ATP (26–30). These physically distinct structures suggest that a specific activating factor for F_0F_1 -ATP synthase must exist. Combined with the findings from this study, we hypothesize that G0s2 may lower the activation barrier of the F_0F_1 -ATP synthase nanomotor and enhance the ATP production rate with the equivalent proton motive driving force (PMF; i.e., the sum of the membrane potential and the pH gradient). Activation barriers might be generated by various factors, such as friction between the stator and rotor of F_0F_1 -ATP synthase, physical and electrical resistance to proton transport through the channel, and the existence of rotary blockers such as the bacterial ϵ -subunit and cyclophilin D (31). The increased ATP production rate caused by G0s2 overexpression observed in the MASC assay supports this hypothesis, because the PMF in the initial phase of this assay should be the same. If this hypothesis is true, even with reduced PMF, cells that express G0s2 should produce ATP faster than cells that express

little or no G0s2. In fact, G0s2 overexpression attenuated the decline of $[ATP]_{mito}$ under hypoxic conditions that reduced the PMF. Precise real-time measurement of the PMF is currently difficult, but these hypotheses might be proven in future studies. Kinetically faster ATP production should accompany greater consumption of both O_2 and PMF; however, our results suggest that preserving ATP production is more beneficial than preserving PMF for cell viability, particularly when the O_2 supply is restricted but still exists. The transience of endogenous G0s2 expression induced by hypoxia might serve to protect tissues in the early phase of energy crisis. There may be specific mechanisms to decrease G0s2 expression under prolonged ischemia that have yet to be identified. Another possible mechanism by which G0s2 could increase the ATP production rate is that G0s2 increases the F_0F_1 coupling efficiency of F_0F_1 -ATP synthase. However, this hypothesis is less likely, because G0s2 altered the oxygen consumption rate to increase the ATP production rate. Although this uncoupling phenomenon has rarely been reported for mammalian mitochondrial F_0F_1 -ATP synthase, we cannot completely eliminate the possibility that intrinsically

uncoupled F_1F_1 -ATP synthase exists, because we could not accurately measure the amount of uncoupled F_1F_1 -ATP synthase in intact cells.

G0s2 was first identified in cultured monocytes during the drug-induced cell cycle transition from G0 to G1 phase (18, 32). A limited number of studies have implied that G0s2 is involved in cell proliferation (33), differentiation (19), apoptosis (34), inflammation (35), and lipid metabolism (36) in various cellular settings. Moreover, G0s2 was reported to localize to the cytosol (33), endoplasmic reticulum (19), mitochondria (34), or the surface of lipid droplets (36). How G0s2 distinguishes these multiple functions is still not clear. In our hands, G0s2 is always localized to mitochondria, which was shown by immunostaining with two antibodies against different epitopes of G0s2 (Fig. S6). Complete depletion of mitochondrial staining by G0s2 knockdown strongly suggests the specific localization of G0s2 to mitochondria. We also showed that G0s2 specifically bound to mitochondrial F_1F_1 -ATP synthase but not other OXPHOS protein complexes and functionally regulated OXPHOS activity. Together, these data suggest that G0s2 acts in the mitochondria. However, different cellular conditions may change the localization and role of G0s2. Additionally, G0s2-mediated changes in ATP metabolism may possibly affect the lipid metabolism or cellular proliferation. Additional studies will reveal the functional mechanisms by which G0s2 exerts these multiple functions in different cellular conditions.

In this study, we evaluated $[ATP]_{mito}$ and $[ATP]_{cyto}$ separately using FRET-based ATP biosensors in living cells. This dual evaluation revealed that $[ATP]_{mito}$ reflected mitochondrial ATP production with much greater sensitivity than $[ATP]_{cyto}$ (Fig. 1 and Movies S1 and S2). Because $[ATP]_{cyto}$ is strongly influenced by the activity of various cytosolic ATP hydrolytic enzymes and

ATP buffering enzymes, $[ATP]_{cyto}$ does not always reflect the ATP availability that determines cellular function.

Taken together, our results indicate that G0s2 is a positive regulator of OXPHOS that works to increase the mitochondrial ATP production rate even under hypoxic conditions. Therefore, enhancing the level and function of G0s2 could be beneficial for hypoxia- and mitochondria-related disorders, such as ischemic diseases, metabolic diseases, and cancer.

Materials and Methods

Cells were infected with adenovirus encoding FRET-based ATP indicators AT1.03 or mit-AT1.03 to measure changes in cytosolic or mitochondrial ATP concentrations, respectively. Image acquisitions and FRET analyses were performed as described previously with some modifications (13). For the control of oxygen concentration during time-lapse imaging, digital gas mixer for stage-top incubator GM8000 (Tokai Hit) was used to create hypoxic (1% O_2) or normoxic (20% O_2) condition. Additional methods are found in *SI Materials and Methods*.

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Liposomal Amiodarone Augments Anti-arrhythmic Effects and Reduces Hemodynamic Adverse Effects in an Ischemia/Reperfusion Rat Model

Hiroyuki Takahama · Hirokazu Shigematsu · Tomohiro Asai · Takashi Matsuzaki · Shoji Sanada · Hai Ying Fu · Keiji Okuda · Masaki Yamato · Hiroshi Asanuma · Yoshihiro Asano · Masanori Asakura · Naoto Oku · Issei Komuro · Masafumi Kitakaze · Tetsuo Minamino

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Abstract

Purpose Although amiodarone is recognized as the most effective anti-arrhythmic drug available, it has negative hemodynamic effects. Nano-sized liposomes can accumulate in and selectively deliver drugs to ischemic/reperfused (I/R) myocardium, which may augment drug effects and reduce side effects. We investigated the effects of liposomal amiodarone on lethal arrhythmias and hemodynamic parameters in an ischemia/reperfusion rat model.

Methods and Results We prepared liposomal amiodarone (mean diameter: 113 ± 8 nm) by a thin-film method. The left coronary artery of experimental rats was occluded for 5 min followed by reperfusion. Ex vivo fluorescent imaging revealed

that intravenously administered fluorescent-labeled nano-sized beads accumulated in the I/R myocardium. Amiodarone was measurable in samples from the I/R myocardium when liposomal amiodarone, but not amiodarone, was administered. Although the intravenous administration of amiodarone (3 mg/kg) or liposomal amiodarone (3 mg/kg) reduced heart rate and systolic blood pressure compared with saline, the decrease in heart rate or systolic blood pressure caused by liposomal amiodarone was smaller compared with a corresponding dose of free amiodarone. The intravenous administration of liposomal amiodarone (3 mg/kg), but not free amiodarone (3 mg/kg), 5 min before ischemia showed a significantly reduced duration of lethal arrhythmias (18 ± 9 s) and mortality (0 %) during the reperfusion period compared with saline (195 ± 42 s, 71 %, respectively).

Conclusions Targeting the delivery of liposomal amiodarone to ischemic/reperfused myocardium reduces the mortality due to lethal arrhythmia and the negative hemodynamic changes caused by amiodarone. Nano-size liposomes may be a promising drug delivery system for targeting I/R myocardium with cardioprotective agents.

Keywords Liposome · Amiodarone · Lethal arrhythmia · Ischemia · Reperfusion

T. Matsuzaki · S. Sanada · H. Y. Fu · K. Okuda · M. Yamato · Y. Asano · I. Komuro · T. Minamino (✉)
Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: minamino@cardiology.med.osaka-u.ac.jp

H. Takahama · M. Asakura · M. Kitakaze
Department of Cardiovascular Medicine, National Cerebral and Cardiovascular Center, Suita 565-8565, Japan

H. Shigematsu · T. Asai · N. Oku
Department of Medical Biochemistry and Global COE, University of Shizuoka Graduate School of Pharmaceutical Sciences, Shizuoka 422-8526 Shizuoka, Japan

H. Asanuma
Department of Cardiovascular Science and Technology, Kyoto Prefectural University School of Medicine, Kyoto 602-8566, Japan

H. Takahama
Division of Cardiovascular Disease, Mayo Clinic, Rochester, MN 55902, USA

Introduction

Therapies for the prevention and treatment of ischemia-induced life-threatening arrhythmias remain an unmet medical need [1]. Amiodarone is currently considered to be the most effective anti-arrhythmic drug available for treating life-threatening arrhythmias [2, 3], despite the fact that this compound has a negative impact on hemodynamic parameters [4, 5]. The intravenous administration of amiodarone is expected

to be beneficial for the immediate treatment of arrhythmias in emergency settings, such as acute myocardial infarction (AMI) [6, 7]. However, in clinical practice, the administration of amiodarone remains problematic for the treatment of AMI [8]. Although lower doses of amiodarone result in fewer incidences of death, high doses of amiodarone can cause hypotension and non-cardiac death, both of which may diminish the positive effects of amiodarone [8, 9]. Therefore, a novel delivery system is strongly desired to enhance the anti-arrhythmic effects of amiodarone without producing severe side effects.

Liposomes are widely used for drug delivery to actively or passively target specific organs and to improve drug stability in cancer and inflammatory diseases [10–12]. In ischemic/reperfused (I/R) myocardium, cellular permeability is enhanced and vascular endothelial integrity is disrupted [13, 14], suggesting that nanoparticles, such as liposomes, may be a promising drug delivery system for targeting I/R myocardium with cardioprotective agents [15]. Indeed, we have recently demonstrated that adenosine encapsulated by liposomes coated with polyethylene glycol (PEG) exhibited enhanced cardioprotective effects and attenuated side effects, such as hypotension and bradycardia, in an ischemia/reperfusion model of rats [16]. In the present study, we prepared liposomal amiodarone and examined 1) the targeted accumulation of liposomal amiodarone in the I/R myocardium, 2) the hemodynamic effects of the intravenous administration of liposomal amiodarone and free amiodarone, and 3) the anti-arrhythmic effects of these preparations in an I/R rat model. We showed that targeting the delivery of liposomal amiodarone to I/R myocardium reduces the mortality due to lethal arrhythmias and the negative hemodynamic changes caused by amiodarone in an I/R rat model.

Methods

Materials

The materials used to prepare PEGylated liposomes, including 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG2000), were kindly donated by Nippon Fine Chemical Co. (Takasago, Hyogo, Japan). Fluorescent beads (diameter 100 nm) were purchased from Invitrogen. All other materials were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Wistar rats (9 weeks old and weighing 250–310 g; Japan Animals, Osaka, Japan) were used. The animal experiments were approved by the Osaka University Research Committee

and were performed according to institutional guidelines. All studies conformed to the Guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Preparation of PEGylated Liposomes

PEGylated liposomes composed of POPC, DPPC, cholesterol, DSPE-PEG2000, and amiodarone were prepared by a thin-film method. Briefly, amiodarone and lipids dissolved in chloroform were evaporated to form a thin lipid film using a rotary evaporator. The lipid film was dried for at least 1 h under reduced pressure and then hydrated with PBS (pH 7.4). The liposome solution was freeze-thawed for 3 cycles with liquid nitrogen. The particle size of the liposomes was adjusted by extrusion through 100-nm-pore polycarbonate filters (Nuclepore, Cambridge, MA, USA). The liposomal solutions were centrifuged at 453,000 g for 15 min (CS120GXL, Hitachi, Japan) to remove the untrapped amiodarone. Then, the liposomes were resuspended in PBS. To determine the efficacy of trapping amiodarone in the liposomes, an aliquot of the liposomal solution was solubilized with 1 % reduced Triton X-100 (Sigma-Aldrich), and the amount of amiodarone was optically determined at 240 nm.

Characterization of PEGylated Liposomes

The particle size and ζ potential of PEGylated liposomes diluted with PBS were measured by dynamic scatter analysis (Zetasizer Nano ZS; Malvern, Worcestershire, UK). The analyses were performed 15 times per sample, and the results represent the analysis of 3 independent experiments.

Experimental Protocol

Targeted Delivery of Fluorescent-labeled Nano-sized Beads to the I/R Myocardium

The rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Catheters were advanced into the femoral vein to infuse the drugs. Ischemia/reperfusion was induced by 5 min of left coronary artery occlusion followed by reperfusion [16]. After the hemodynamic parameters became stable, fluorescent-labeled nano-size beads, 100 nm in diameter (FluoSpheres, Invitrogen), were intravenously infused to the rats for 5 min before ischemia or before a sham operation ($n=3$, each). Fifteen minutes after reperfusion, the hearts were removed and cut into 5 sections parallel to the axis from the base to the apex. Then, ex vivo fluorescence images were obtained with an Olympus SZX12 stereoscopic microscope equipped with a DP71 digital camera (Olympus, Tokyo, Japan) before and after the hearts were sliced.

Targeted Delivery of Amiodarone and Liposomal Amiodarone to the I/R Myocardium

Catheters were advanced into the femoral artery and vein to measure the systemic blood pressure (BP) and to infuse the drugs into the anesthetized rats, respectively. Electrocardiographic and hemodynamic parameters, such as heart rate (HR) and BP, were continuously monitored during the study using a PowerLab system (ADInstruments, Castle Hill, Australia). After the hemodynamic parameters became stable, to clarify the targeted delivery of amiodarone and liposomal amiodarone to the I/R myocardium, we intravenously administered saline, free amiodarone (3 mg/kg) or liposomal amiodarone (3 mg/kg) to rats for 5 min before the onset of ischemia. Then, we obtained blood samples and myocardium from the I/R area.

Effects of Amiodarone and Liposomal Amiodarone on Lethal Arrhythmias

To evaluate the effects of amiodarone and liposomal amiodarone on lethal arrhythmias, we intravenously administered saline ($n=7$), free amiodarone (3.0 or 10.0 mg/kg) ($n=6$ each), PEGylated liposomes (empty liposomes) ($n=6$), and PEGylated liposomal amiodarone (3.0 mg/kg) ($n=6$) for 5 min before ischemia. The dose of amiodarone used in this study was lower than that used in a previous study [17] to clarify whether amiodarone encapsulated by liposomes coated with PEG exhibited enhanced anti-arrhythmic effects. Without any procedure such as electrical conversion or cardiac massage, ventricular tachyarrhythmias (VT/VF) occurred frequently during early period of reperfusion and the mortality of rats reached more than a half of cases in this model [18].

Measurement of Amiodarone Concentration

The concentration of amiodarone in serum and heart tissue from the I/R area was assayed by high-performance liquid chromatography (HPLC) as previously described [19]. The detection limit of the HPLC assay was 50 ng/mL. Blood and myocardial samples were obtained at the end of the experimental protocol. The sample preparation was performed as previously described [19]. Briefly, myocardium was freed from visible blood, thereafter rinsed with 0.9 % sodium chloride and stored at -20°C until analysis. After that, myocardial tissue samples were finely minced and 100 mg were homogenized with 0.9 % sodium chloride (1 mL) and after centrifugation, the clear supernatant was injected into HPLC.

Quantitative Evaluation of Fluorescent-labeled Nano-sized Beads in the I/R Myocardium

To analyze the quantitative fluorescent intensity, signals from heart slices were quantified by image analysis (Image

J; National Institutes of Health, USA) as previously described [20]. The signal intensity from the heart slices was evaluated as the average signals of the whole heart and the left ventricle (LV) (Fig. 2c).

Arrhythmia Analysis

The electrocardiographic tracings were independently analyzed by two of the authors, who were blinded to the treatment assignment. The duration of each spontaneous ventricular tachycardia or fibrillation episode during the I/R protocol was measured using the time scale provided by the recording software. Ventricular tachycardia was defined as 4 or more consecutive ventricular ectopic beats, and ventricular fibrillation was defined as a signal in which the individual QRS deflections could not easily be distinguished from one another. However, distinguishing ventricular tachycardia from fibrillation was often difficult [21]; therefore, we report ventricular tachycardia and fibrillation collectively as ventricular tachyarrhythmias (VT/VF) in this study. VT/VF duration and mortality were evaluated for 5 min of ischemia followed by 15 min of reperfusion.

Statistical Analysis

The parameters of the liposomes are expressed as the mean \pm standard deviation (SD). Other data are expressed as the average \pm standard error of the mean (SEM). To compare the parameters of the liposomes, unpaired *t*-tests were performed. We performed the Welch *t*-test to compare the amiodarone concentration in the plasma and myocardium. For hemodynamic parameters, the data were assessed with the paired *t*-test for comparisons to the baseline within a group. One-way repeated-measurement ANOVA followed by post-hoc Bonferroni's multiple comparisons were used for comparisons between groups. To address the differences in VT/VF duration among the groups, we performed a non-parametric (Kruskal-Wallis) test followed by evaluation with the Mann-Whitney *U* test. The mortality rates were compared using the Fisher's exact probability test. In all analyses, $P<0.05$ was considered to be statistically significant.

Results

Characterization of PEGylated Liposomes

We prepared 5 types of PEGylated liposomes composed of POPC, DPPC, cholesterol, and amiodarone. The ratio of unsaturated lipids (POPC) to saturated lipids (DPPC) varied (Fig. 1). During preparation of the liposomes, the POPC:DPPC:cholesterol:amiodarone molar ratio of 10:0:5:1 exhibited the best encapsulation efficiency for amiodarone compared with the other conditions (Fig. 1).

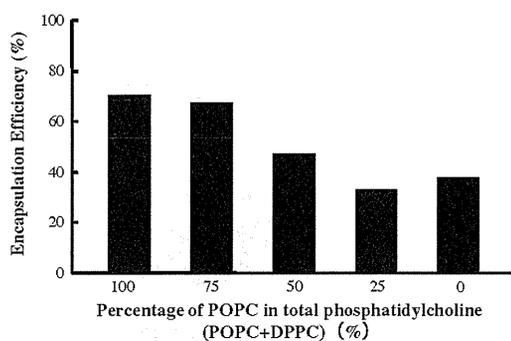


Fig. 1 Encapsulation efficiency of amiodarone in the liposomes. Amiodarone was loaded into liposomes containing POPC, DPPC, or a mixture of POPC and DPPC. The liposomal amiodarone was composed of phosphatidylcholine (POPC + DPPC):cholesterol:amiodarone at a 10:5:1 molar ratio. The percent molar ratio of POPC in total phosphatidylcholine (POPC + DPPC) is indicated in the figure. The encapsulation efficiency of amiodarone was determined as described in the Methods section

The dynamic light scatter analysis showed no significant differences between the mean diameter, polydispersity index, or ζ potential distribution of the empty and amiodarone-loaded PEGylated liposomes (Table 1).

Accumulation of Fluorescence-labeled Nano-sized Beads in the I/R Myocardium

Representative pictures obtained by fluorescence imaging are shown in Fig. 2a (whole heart) and b (sliced hearts). Quantitative analysis revealed that the average fluorescence intensity of the whole heart (Fig. 2c left) or the left ventricle (Fig. 2c right) of the I/R hearts was significantly higher than that in sham-operated hearts.

Amiodarone Concentration in the Blood and I/R Myocardium

The plasma concentration after the administration of liposomal amiodarone was significantly higher than that of free amiodarone (Table 2). Importantly, the amiodarone concentration in the I/R myocardium was detectable after the administration of liposomal, but not free, amiodarone (Table 2).

Hemodynamic Effects of Amiodarone and Liposomal Amiodarone

The baseline heart rates were 411 ± 16 , 426 ± 14 , 427 ± 12 , 409 ± 8 and 414 ± 6 beats/min in the saline, empty liposome, amiodarone (3 mg/kg), amiodarone (10 mg/kg) and liposomal amiodarone (3 mg/kg) groups, respectively. The baseline systolic BP was 113 ± 7 , 118 ± 10 , 111 ± 5 , 90 ± 4 and 104 ± 2 mmHg in the saline, empty liposome, amiodarone (3 mg/kg), amiodarone (10 mg/kg) and liposomal amiodarone (3 mg/kg) groups, respectively. There were no significant differences in the baseline HR or systolic BP among the groups tested. The intravenous administration of amiodarone (3 and 10 mg/kg) or liposomal amiodarone reduced both the HR and systolic BP from the baseline, whereas the saline or empty liposomes did not (Fig. 3). The time-course changes of both the HR and systolic BP were significantly smaller in the liposomal amiodarone group (3 mg/kg) compared with the corresponding dose in the free amiodarone group (3 mg/kg) (Fig. 3). The reductions in HR and systolic BP at 1, but not 3, minutes after liposomal amiodarone administration were significantly smaller compared with those following the corresponding dose of amiodarone.

Antiarrhythmic Effects of Amiodarone and Liposomal Amiodarone

Representative electrocardiograms of the rats that received saline, free amiodarone or liposomal amiodarone are shown in Fig. 4. The intravenous administration of liposomal amiodarone (3 mg/kg), but not amiodarone (3 mg/kg), significantly reduced the duration of VT/VF compared with saline (Table 3). Furthermore, the mortality in the group that received liposomal amiodarone (3 mg/kg), but not the corresponding dose of amiodarone (3 mg/kg), was significantly lower than that in the saline group. In the group of rats that received a high dose of amiodarone (10 mg/kg), the VT/VF duration was 36 ± 12 s, and none of the rats died (Table 3), which was similar to the low dose of liposomal amiodarone group (3 mg/kg).

Discussion

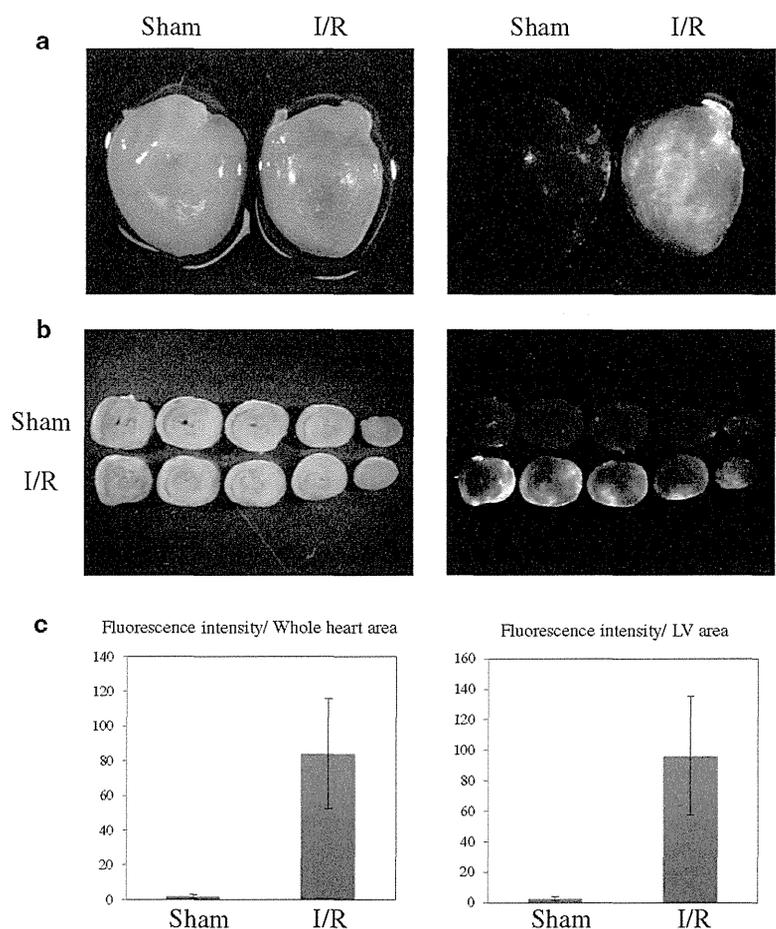
In this study, we revealed that 1) liposomal amiodarone was successfully prepared using a thin-film method, 2) the

Table 1 Characterization of liposomes by dynamic light scatter analysis

	Mean diameter (nm)	Polydispersity index	ζ Potential (mV)
PEGylated liposomes (empty liposomes)	111 ± 14	0.124 ± 0.027	-2.1
PEGylated liposomal amiodarone	113 ± 8	0.128 ± 0.040	-3.7

Results represent 4 independent experiments. The values are expressed as the mean \pm SD. PEG polyethylene glycol

Fig. 2 Representative pictures of ischemia/reperfused myocardium with and without fluorescence-labeled nano-sized beads. Representative pictures obtained by fluorescent imaging are shown in **a** (*whole heart*) and **b** (*sliced hearts*). Quantitative analysis revealed that the average fluorescence intensity of the whole heart (**c left**) or the left ventricle (**c right**) of the I/R hearts was significantly higher than that of the sham-operated hearts



accumulation of nano-sized beads was observed in the I/R myocardium, 3) liposomal amiodarone showed a smaller reduction in the HR and systolic BP compared with free amiodarone, and 4) liposomal amiodarone, but not amiodarone, reduced the VT/VF duration and mortality during the reperfusion period compared with saline.

Table 2 Amiodarone concentration in the blood and I/R myocardium

Groups	Plasma, ng/mL	Myocardium, ng/mL
Saline	N.D.	N.D.
Free amiodarone	472±147	N.D.
Liposomal amiodarone	3872±378*	71±7*

Data are expressed as the mean ± SEM. N.D. not detected. $n=3$ rats in each group. * $p<0.05$ versus free amiodarone

Preparation of Liposomal Amiodarone

This study is the first to encapsulate amiodarone in PEGylated liposomes, although it has been previously encapsulated in other liposomes [22] and micelles [23]. We demonstrated that lipid bilayers composed of unsaturated lipids are more suitable for encapsulating amiodarone in PEGylated liposomes compared with those composed of saturated lipids. PEGylated liposomes have a long circulating time in the bloodstream because PEG endows a steric barrier to liposomes, allowing them to avoid interactions with opsonins and cells of the mononuclear phagocytic system [24]. Thus, they have been used to increase drug stability, safety, and bioavailability in clinical applications. In this study, we found that a higher concentration of amiodarone was retained in the blood when we administered liposomal amiodarone compared with the administration of

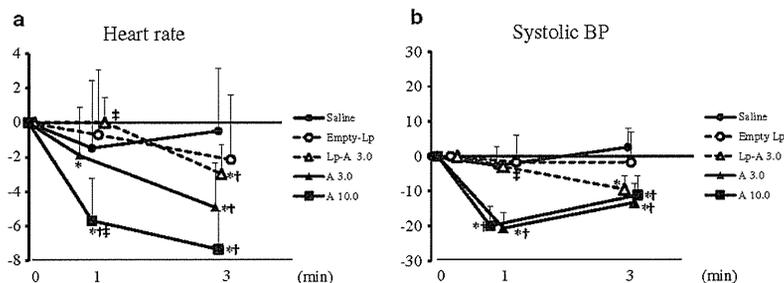


Fig. 3 Time-course changes in HR and systolic BP after drug administration. Shows the percent change from baseline for HR (a) and systolic BP (b) after intravenous administration of the tested drugs. The data are expressed as the mean \pm SEM. * P <0.05 versus baseline, paired t -test. P =0.0009 (HR), 0.0002 (systolic BP) between

amiodarone (3 mg/kg) and liposomal amiodarone (3 mg/kg), 1-way repeated-measurement ANOVA. † P <0.05 versus saline, ‡ P <0.05 versus amiodarone (3 mg/kg), 1-way repeated-measurement ANOVA with Bonferroni's multiple comparison

free amiodarone, suggesting that encapsulation of amiodarone in PEGylated liposomes enhances the stability of amiodarone in the blood.

Targeted Delivery to the I/R Myocardium by Liposomal Amiodarone

Ex vivo fluorescence imaging revealed that fluorescence-labeled nano-sized beads accumulated in the I/R myocardium, suggesting that myocardial permeability can be enhanced in the I/R myocardium. Consistent with this finding, we

observed that the amiodarone concentration in the I/R myocardium in the liposomal amiodarone group was much higher compared with that in the amiodarone group. Enhanced permeability in the I/R myocardium and the prolonged presence of amiodarone in PEGylated liposomes in the blood represent a possible mechanism for increased amiodarone concentrations in the I/R myocardium. Amiodarone will be released from accumulated liposomal amiodarone in I/R myocardium due to the natural decay and concentration gradient. These findings suggest that the I/R myocardium is a promising passive target for liposomal drug delivery.

Fig. 4 Representative electrocardiograms. The upper, middle and lower panels show representative electrocardiograms under baseline conditions during ischemia and at the onset of reperfusion for rats that received saline, free amiodarone (3 mg/kg) and liposomal amiodarone (3 mg/kg), respectively

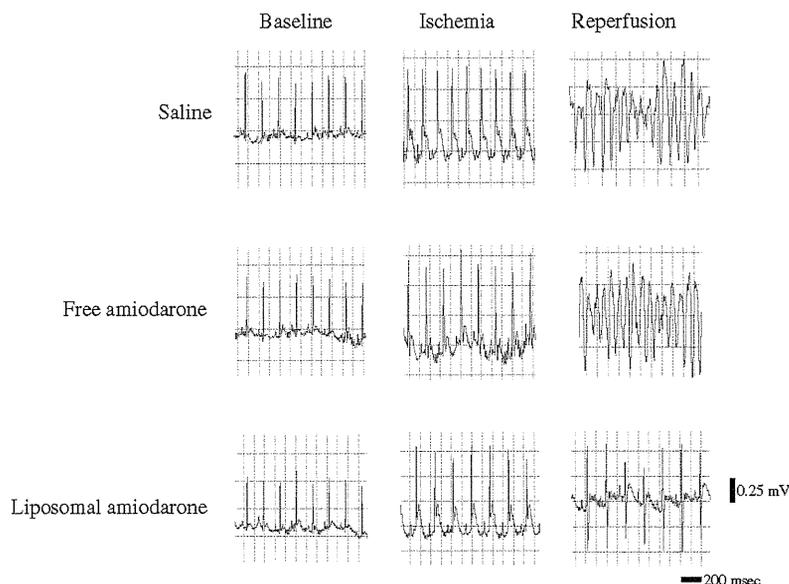


Table 3 Lethal arrhythmias and mortality in an I/R rat model

	Number	VT/VF duration (sec)	Mortality (%)
Saline	7	195±42	71
Empty liposomes	6	162±31	50
Amiodarone (3 mg/kg)	6	167±78	33
Amiodarone (10 mg/kg)	6	36±12*	0#
Liposomal Amiodarone (3 mg/kg)	6	18±9*	0#

* $p < 0.05$ versus saline (VT/VF duration). # $p < 0.05$ versus saline group (mortality). VT ventricular tachycardia, VF ventricular fibrillation

Minimal Negative Hemodynamic Effects of Liposomal Amiodarone

Amiodarone causes hypotension and bradycardia in clinical settings [4, 5]. In this study, both free and liposomal amiodarone significantly reduced the HR and systolic BP; however, the time-course changes for both the HR and systolic BP in the liposomal amiodarone group were significantly smaller compared with those following the corresponding dose of free amiodarone. Importantly, the reductions in HR and systolic BP at 1, but not 3, minutes after liposomal amiodarone administration were significantly smaller compared with those following the corresponding dose of amiodarone. These findings suggest that liposomal amiodarone may minimize the negative effects on systemic hemodynamics immediately after the administration of amiodarone. One possible mechanism to explain this finding is that amiodarone on the surface of the liposome membrane is covered with PEG so that amiodarone cannot act directly on cardiovascular cells. Gradual release of amiodarone from liposome may minimize the rapid hemodynamic changes, because systemic hemodynamic effects of liposomal amiodarone were significantly attenuated in liposomal amiodarone group than free amiodarone group.

Augmented Anti-arrhythmic Effects of Liposomal Amiodarone

In this study, liposomal amiodarone (3 mg/kg), but not the corresponding dose of free amiodarone (3 mg/kg), significantly reduced the VT/VF duration and mortality compared with saline in an I/R rat model. Because the acute effects of amiodarone are known to be attributable to blockade of Na^+ , Ca^{2+} and dose-dependent K^+ channels [2, 25], increasing the concentration of amiodarone in the I/R myocardium may augment its anti-arrhythmic effects through its tonic effects on cardiomyocytes caused by blocking cardiac ionic currents. Kishida et al. reported that amiodarone enhances nitric oxide production in cultured human endothelial cells [26].

Furthermore, amiodarone protects cardiac myocytes against oxidative injury by scavenging free radicals [27]. These pleiotropic effects of amiodarone are also enhanced by its increased concentration in the I/R myocardium via PEGylated liposomes, which may contribute to the reduction of lethal arrhythmias during reperfusion followed by ischemia. In the present study, since we did not do any procedure such as electrical conversion or cardiac massage for VT/VF, the mortality was higher than in our previous report [16].

Clinical Implications

In clinical settings, higher doses of amiodarone cause hypotension and non-cardiac death or induce worsening heart failure through negative inotropic effects [28]. These effects often diminish the beneficial effects of amiodarone for patients with AMI or heart failure [8, 9]. The present study demonstrated that liposomal amiodarone (3 mg/kg) exerts anti-arrhythmic effects similar to a high dose of free amiodarone (10 mg/kg) while reducing the extent of bradycardia and hypotension, suggesting that encapsulating amiodarone in liposomes augments its anti-arrhythmic effects and reduces its negative effects on hemodynamic parameters with reducing administrative dose. These findings can have a great impact on preventing lethal arrhythmias during reperfusion in AMI patients.

Study Limitations

There are several limitations in this study. We used a brief period of I/R without myocardial infarction in rats. Sakamoto et al. demonstrated that the incidence of VT/VF in a rodent model was 'bell-shaped' with a maximum at 5 min of ischemia and that most lethal arrhythmias occurred within first 20 s after the onset of reperfusion [29]. Consistently, our data showed that the mean time at which the lethal arrhythmia occurred after the onset of reperfusion was 3.3 ± 1.6 s. Therefore, we chose the 5 min of ischemia followed by 15 min of reperfusion model. We also chose the timing of drug administration before the onset of ischemia to clarify whether liposomal-amiodarone could prevent the lethal arrhythmia that occurs in the early period of reperfusion. In addition, in clinical practice lethal arrhythmias often occur after a brief period of I/R without any irreversible damage to the heart, indicating that the anti-arrhythmic effects of liposomal amiodarone during a brief period of ischemia model could have clinical relevance [30]. However, careful interpretation is necessary when using liposomal amiodarone in acute myocardial infarction with irreversible damage to confirm the beneficial effects of liposomal amiodarone. Furthermore, because the electrophysiology of rats differs from that of humans and drug administration in our study started before the onset of

ischemia, additional pre-clinical studies including a longer period of I/R model to consider the timing of drug administration are needed using large animal models. We should also take into account that the potential side effects of amiodarone such as bradycardia are minimal in the left coronary artery occlusion model used in the present study.

Conclusion

In conclusion, the targeted delivery of liposomal amiodarone to the I/R myocardium exerted strong anti-arrhythmic effects and reduced the negative impact on systemic hemodynamics. Nano-sized liposomes may be a promising drug delivery system for targeting the I/R myocardium with cardioprotective agents.

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