

Fig. 6 G-CSF increased the number of CD31(+) cells in the infarcted area.

(A-D) Epicardial and endocardial layer of the infarcted area was separately shown in the control and G-CSF treated groups. Green and blue signals indicated CD31 and nuclei, respectively. Scale bars show 50 μ m. (E, F) The number of CD31(+) cells of the infarcted area was obtained with immunofluorescent staining, quantitated by NIH image and shown in E and F. CD31(+) cell density of the epicardial layer in G-CSF group was significantly higher than that of the control. CD31(+) cell density of the endocardial layer in G-CSF group has a tendency to be higher than that the control group, but was not statistically significant.

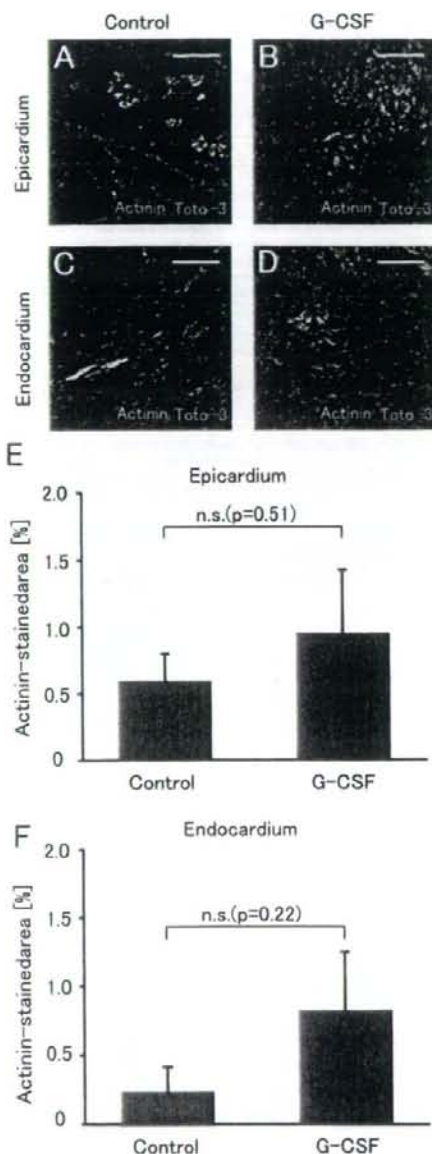


Fig. 7 Immunofluorescent staining for actinin on the infarcted area

(A)~(D) Representative immunofluorescent microphotograph of the infarcted area using cardiac actinin antibody. (A, B) Comparison of the endocardium between control (A) and G-CSF group (B). (C, D) Comparison of the epicardium between control (C) and G-CSF group (D). Blue = Nuclei stained by TOTO-3. Scale bars show 50 μ m. (E, F) The actinin-positive cell density in the endocardium (E) and the epicardium (F). The actinin-positive cell density of G-CSF group had a tendency to be greater than that of the control group in both endocardium and epicardium, although it was not statistically significant.

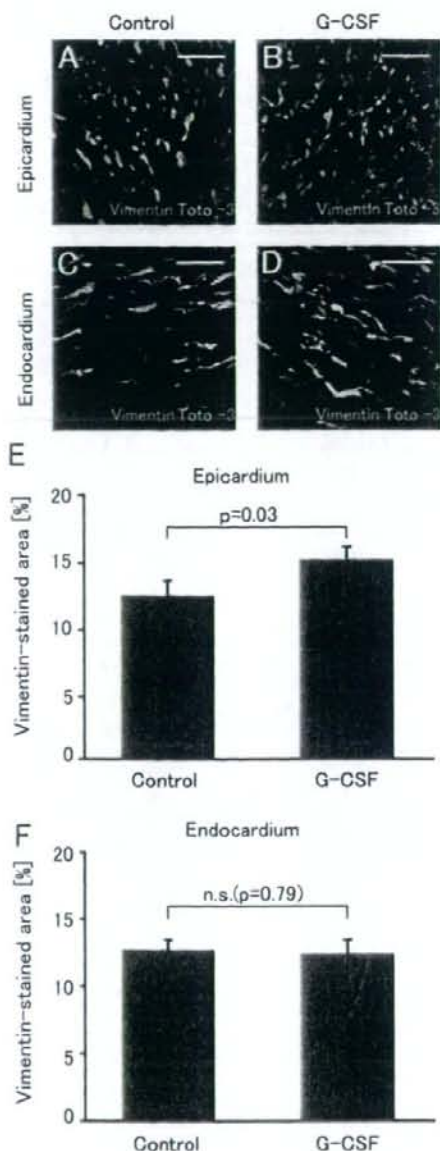


Fig. 8 Immunofluorescent staining for vimentin on the infarcted area

(A)–(D) Representative immunofluorescent microphotograph of the infarcted area using anti-vimentin antibody. (A, B) Comparison of the endocardium between control (A) and G-CSF group (B). (C, D) Comparison of the epicardium between control (C) and G-CSF group (D). Blue = Nuclei stained by TOTO-3. Scale bars show 50 μ m. (E, F) The vimentin-positive cell density in the endocardium (E) and the epicardium (F). The vimentin-positive cell density of G-CSF group had a tendency to be greater than that of the control group in epicardium, although it was not statistically significant.

clearly expressed α -SMA and vimentin, indicating that they were myofibroblasts. Some also weakly expressed CD45. In the chronic phase of cardiac remodeling, GFP⁺ fibroblast-like cells were still present in the scar tissue of the heart. These cells were positive for vimentin, but most of them were negative for α -SMA. These results indicated that hematopoietic stem cell-derived cells migrated into the infarcted area, and differentiated into myofibroblasts in the acute phase, and became mature fibroblasts by the chronic phase. G-CSF treatment was associated with an increased number of infiltrating myofibroblasts in both groups, as well as a decrease in mortality and an improvement cardiac function in the chronic phase. Moreover, we found that hematopoietic stem cell-derived cells (possibly monocytes) contributed the accumulation of myofibroblasts at the perivascular fibrosis area in pressure overload-induced cardiac hypertrophy model.²¹ These results implicate the infiltration of myofibroblasts as critical in the prevention of cardiac remodeling.

The present study showed that G-CSF significantly increased the thickness of the infarcted wall. Moreover, we found that G-CSF significantly increased the cell density of the endothelial cells, α -SMA positive cells and vimentin positive cells but not the cardiomyocytes. However, both α -SMA and vimentin is not a specific marker for special cell types. The α -SMA was expressed in various cell types such as smooth muscle cells, cardiac myofibroblasts, and juvenile cardiomyocytes. Vimentin was mainly expressed in myofibroblasts, but was expressed in other cell types. From the finding of the cell morphology, we supposed that some of α -SMA positive cells might be vascular smooth muscle cells. We also think that most of the cells positive for α -SMA and vimentin might be myofibroblasts, the origin of which might be the mobilized monocytes by G-CSF administration. Taken together, the present findings indicated that G-CSF increased the BM-derived cells in the infarcted area, which may play an important role in modulating post-MI remodeling. This phenomenon was basically in accordance with our previous observation obtained from BM transplanted mice. A number of cell transplantation experiments revealed that fibroblasts, smooth muscle cells, cardiomyocytes and even skeletal muscle cells improved cardiac remodeling and function when they were transplanted into the infarcted area after MI.^{6,23–28} The increased cellular density might be critically involved in the healing process of myocardial infarction by secreting extracellular matrix, angiogenic growth factors and cytokines, which may accelerate healing process. Previous papers reported the molecular mechanism of the action of G-CSF.

Recently, Harada reported that G-CSF directly binds to G-CSF receptors on cardiomyocytes, activating the JAK/STAT pathway, and preventing apoptosis in the late

phase after MI. This effect was proposed to improve cardiac remodeling.²⁹ An anti-apoptotic effect on cardiomyocytes might also play a role in the improvement of cardiac remodeling mediated by G-CSF in our study. In addition, endothelial progenitor cells might also play a role in the regeneration of the infarcted myocardium in the chronic phase. Cardiac progenitor cells have also been shown to be resident in the heart,³⁰ and we supposed that they also participate in the healing process after MI. These findings indicated that these mechanisms may collaboratively work and improved the cardiac function. A great deal of clinical research up until 2005 involved harvesting mononuclear cells from BM or peripheral blood and infusing them through a catheter into a coronary artery to treat acute myocardial infarction. Strauer and colleagues first reported transplantation of BM mononuclear cells 4.8-13.5 days after a myocardial infarction in 10 acute myocardial infarction patients, which resulted in a slight decrease in left-ventricular end-systolic dimension and infarct region, and an increase in the left-ventricular ejection fraction and regional function.⁶ The TOPCARE-AMI trial allocated 20 patients with reperfused acute myocardial infarction to receive intracoronary infusion of either BM-derived or circulating blood-derived progenitor cells into the infarct artery at 4.3±1.5 days. The results from this trial indicated a significant increase in global left ventricular ejection fraction, improved regional wall motion, and reduced end-systolic left ventricular volumes at the 4-month follow-up investigation. At the one year follow-up investigation the transplanted group revealed an increased EF, reduced infarct size, and absence of reactive hypertrophy without significant complication, suggesting functional regeneration of the infarcted ventricles³¹⁻³³ (41-43). The BOOST trial, and Fernandes-Aviles and colleagues also transplanted BM mononuclear cells 4.8-13.5 days after a myocardial infarction in acute myocardial infarction patients. In most of the studies, transplantation resulted in similar results.^{34,35} The IACT Study transplanted BM mononuclear cells via catheter in patients with chronic MI, and reported that functional and metabolic regeneration of infarcted and chronically avital tissue could be realized.³⁶

In conclusion, the precise investigation of molecular mechanisms and the double-blind clinical trial will clarify the usefulness of this cytokine therapy for the prevention of cardiac remodeling after myocardial infarction.

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Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia–reperfusion injury

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ABSTRACT

Inhalation of hydrogen (H₂) gas has been demonstrated to limit the infarct volume of brain and liver by reducing ischemia–reperfusion injury in rodents. When translated into clinical practice, this therapy must be most frequently applied in the treatment of patients with acute myocardial infarction, since angioplastic recanalization of infarct-related occluded coronary artery is routinely performed. Therefore, we investigate whether H₂ gas confers cardioprotection against ischemia–reperfusion injury in rats. In isolated perfused hearts, H₂ gas enhances the recovery of left ventricular function following anoxia–reoxygenation. Inhaled H₂ gas is rapidly transported and can reach ‘at risk’ ischemic myocardium before coronary blood flow of the occluded infarct-related artery is reestablished. Inhalation of H₂ gas at combustible levels during ischemia and reperfusion reduces infarct size without altering hemodynamic parameters, thereby preventing deleterious left ventricular remodeling. Thus, inhalation of H₂ gas is promising strategy to alleviate ischemia–reperfusion injury coincident with recanalization of coronary artery.

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Acute myocardial infarction is a leading cause of death worldwide. Reduction of infarct size is an important therapeutic goal, since the size of the infarct is directly linked to short-term and long-term morbidity and mortality [1]. The prognosis of acute myocardial infarction has been improved dramatically with the development of highly successful approaches to restore blood flow by primary percutaneous coronary intervention (PCI) to the ischemic tissue [2]. Paradoxically, while coronary reperfusion improves the prognosis of acute myocardial infarction, it also leads to myocardial reperfusion injury by extending myocardial damage within the ischemic period [3]. Studies in animal models of acute myocardial infarction show that reperfusion injury accounts for up to 50% of the final size of a myocardial infarct [4]. Therefore, intervention to alleviate reperfusion injury at the time of coronary recanalization has been considered to be the promising strategy to further

decrease infarct size and improve the prognosis after myocardial infarction.

The accelerated generation of reactive oxygen species (ROS) by reperfusion of the ischemic myocardium is a potential mediator of reperfusion injury [5–7]. Many attempts have been made to inhibit ROS production to limit the extent of reperfusion injury. However, the administration of ROS scavengers at the time of reperfusion has produced conflicting results [8,9]. That can be partially explained by the dual role of ROS in ischemia–reperfused hearts. The majority of detrimental effects associated with lethal reperfusion injury are attributed to hydroxy radical ([•]OH), the most highly reactive oxygen species. By comparison, superoxide anion radical (O₂^{•-}) and hydrogen peroxide (H₂O₂) have less oxidative energy and, paradoxically, are implicated as crucial signaling components in the establishment of favorable tolerance to oxidative stress upon ischemia–reperfusion [10,11]. Consequently, the inhibition of both pathways can be deleterious.

Recently, Ohsawa et al. demonstrated that molecular hydrogen (H₂) is a novel anti-oxidant with certain unique properties. (1) H₂ is permeable to cell membranes and can target organelles,

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including mitochondria and nuclei; (2) H₂ specifically quenches exclusively detrimental ROS, such as ·OH and peroxynitrite (ONOO⁻), while maintaining the metabolic oxidation-reduction reaction and other less potent ROS, such as O₂⁻, H₂O₂, and nitric oxide (NO⁻); (3) inhalation of H₂ gas limits the infarct volume of brain and liver if given at the appropriate time during reperfusion [12,13]. However, clinical application of reperfusion therapy for these organs is limited. When translated into the clinical practice, H₂ gas inhalation therapy must be most frequently applied in the treatment of patients with acute myocardial infarction, since angioplastic recanalization of occluded infarct-related coronary artery is routinely performed.

The aim of this study was to investigate whether inhalation of H₂ gas exerts cardioprotective effects during myocardial ischemia-reperfusion. We showed the inhaled H₂ gas is rapidly transported and can reach even 'at risk' ischemic myocardium before coronary blood flow of the occluded infarct-related artery is reestablished. Inhalation of H₂ gas during ischemia and reperfusion significantly reduces infarct size without altering hemodynamic parameters, thereby preventing deleterious left ventricular (LV) remodeling.

Materials and methods

Animals. All experimental procedures and protocols were approved by the Animal Care and Use Committees of the Keio University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Eight-week-old male Wistar rats were artificially ventilated under anesthesia with ketamine (60 mg/kg) and xylazine (15 mg/kg) given intraperitoneally. Temperature was maintained at 37.5 ± 0.5 °C using a thermostatically controlled heating blanket connected to a thermometer probe placed in the rectum. H₂ gas was administered through a ventilator and the flow volume was controlled by a gas flowmeter TF-1 (YUTAKA Engineering Corporation, Tokyo, Japan). The concentration of H₂ in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERAMECS, Kyoto, Japan). Saturation of arterial oxygen level (SaO₂) was monitored by Clip sensor (PDR-43C) connected to Stand Alone Pulseoxymeter (CANL425SV). A Millar transducer catheter (SPR-320) was placed in the LV cavity via the left internal artery to monitor LV pressure using Polygraph system (NIHON KODEN; PEG-1000).

Myocardial ischemia-reperfusion model. Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. After 30 min of ischemia, we removed the tube for myocardial reperfusion and closed the thorax with the suture intact. The suture around the coronary artery was retied 24 h after reperfusion and 2% Evans blue dye was injected into the LV cavity to retrospectively delineate the area at risk of myocardial infarction. The heart was removed, washed in phosphate buffered saline, and then sliced into sequential 1 mm thick sections. We stained the sections with 2,3,5-triphenyltetrazolium chloride (TTC) (3%) then measured the infarct (white), non-infarct (red), non-ischemic, (blue), and at risk areas (AAR) (white and red).

Echocardiography. Rats were anesthetized by inhalation with 1.5% isoflurane. Animals were anchored to a positionable platform in a supine position. Short axis echocardiography was accomplished with a Vevo 660 system (VisualSonics) with the use of a 600 series real-time microvisualization scanhead probe.

Measurement of H₂ gas concentration. H₂ gas concentration was measured in tissues using a needle-type H₂ sensor (Unisense). The electrode current was measured with a picoammeter (Keithley) attached to a strip chart. The negative current obtained from the H₂ sensor was converted to regional H₂ concentration using a

calibration curve generated from known levels of H₂ saturated saline.

Langendorff-perfusion of the heart. Hearts were excised quickly from heparinized Wistar male rats (350 g) and perfused with modified Krebs-Henseleit buffer (118 mmol/l NaCl, 25 mmol/l NaHCO₃, 4.7 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 1.75 mmol/l CaCl₂, 0.5 mmol/l EDTA, 11 mmol/l glucose, and 5 mmol/l pyruvate) equilibrated with a gas mixture comprised of 95% O₂/5% CO₂ at 37 °C. Coronary perfusion pressure was maintained at 70 mmHg. A plastic catheter with a latex balloon was inserted into the LV. Before the induction of anoxia, hearts were paced at 5 Hz, and the LV end-diastolic pressure was adjusted to 10 mmHg by filling the balloon with water. Pacing was turned off during anoxia and turn on 10, 20, 30, or 40 min after reoxygenation to measure the recovery of LV function. Indices of LV function [LV systolic pressure, LVSP; LV diastolic pressure, LVDP; LV developed pressure (LVDP = LVSP - LVDP); and LV peak positive and negative dP/dt] were recorded as described previously [14–17].

Immunohistochemical procedures. Sample fixation, embedding, sectioning, and blocking were performed as described previously [18]. Briefly, hearts were perfused from the apex with PBS, perfusion-fixed with 4% paraformaldehyde/PBS, dissected, subsequently cryoprotected in sucrose solutions at 4 °C, embedded in OCT compound (Miles Scientific, Naperville, IL), and quickly frozen in liquid nitrogen. The fixed hearts were sectioned (8 μm) using a CM3050S cryostat (Leica, Nussloch, Germany). For immunostaining, sections were blocked in 5% BSA for 30 min at room temperature and stained with anti-8OH-dG (MOG-020P; Japan Institute for the Control of Aging; 1:800) antibodies overnight at 4 °C. Secondary antibodies conjugated Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA; 1:200) were applied for 1 h at 4 °C. Nuclei were stained with TO-PRO-3 (Molecular Probes) in a mounting medium. Slides were observed under Fluorescence Microscope (LYMPUS BX-60). The 8-OHdG positive area as percentage of total left ventricles at serial short axis sections was measured by planimetry using ImageJ software from the National Institutes of Health (Bethesda, MD, USA).

Statistical analyses. Values are presented as means ± SEM. Statistical significance was evaluated using the unpaired Student's *t*-tests for comparisons between two mean values. Multiple comparisons between more than three groups were performed using ANOVA. A value of *P* < 0.05 was considered statistically significant.

Results

H₂ gas improves the recovery of left ventricular function during reoxygenation after anoxia in isolated perfused hearts

We first studied the effect of H₂ gas on the functional recovery after anoxia-reoxygenation in Langendorff-perfused rat hearts. Hearts were subjected to 40 min of anoxic perfusion with buffer equilibrated with either 100% N₂ (Control group) or 100% H₂ (H₂ group) followed by 40 min of aerobic reperfusion with buffer equilibrated with 95% O₂ and 5% CO₂ (Fig. 1A). H₂ gas significantly improved the recovery of LV developed pressure (LVDP), positive dP/dt, and negative dP/dt 40 min after reoxygenation (*n* = 10, **P* < 0.05, compared to control group, Fig. 1B).

Inhalation of H₂ gas immediately increases the intramyocardial H₂ gas concentration

Before we determined whether inhalation of hydrogen (H₂) gas confers cardioprotection against ischemia-reperfusion injury, the regional delivery of inhaled H₂ gas was investigated by monitoring the time-course of changes in H₂ levels using a needle-shaped

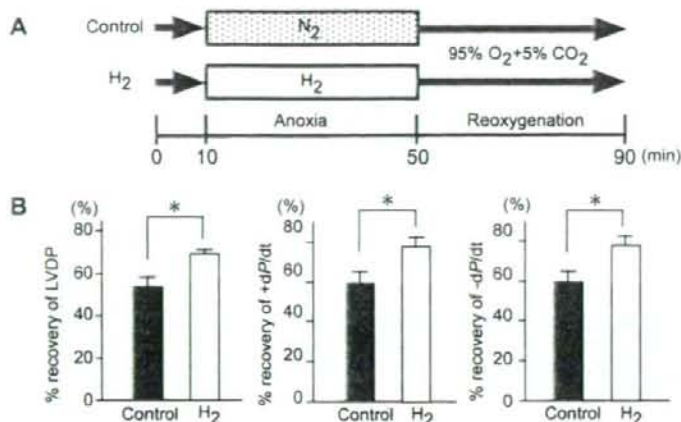


Fig. 1. H₂ gas improves the recovery of left ventricular function during reoxygenation after anoxia in isolated perfused hearts. (A) Experimental protocol of anoxia-reoxygenation. Isolated perfused rat hearts were subjected to 40 min of anoxia with buffer equilibrated with either 100% N₂ (control group) or 100% H₂ (H₂ group) followed by 40 min of aerobic reperfusion. (B) Comparison of percentage recovery of LVDP and peak positive and negative dP/dt 40 min after reoxygenation between control group and H₂ inhalation group ($n = 10$, $P < 0.05$, compared to control group).

hydrogen sensor electrode inserted directly into the tissues. When 2% H₂ gas was inhaled, the arterial H₂ levels started to increase 2 min after inhalation of H₂ gas and reached a maximum level after 5 min [$1.82 \pm 0.02\%$ ($n = 5$)]. The incremental rate of H₂ saturation for the non-ischemic myocardium was similar to that observed in arterial blood with attaining a maximum of $1.73 \pm 0.02\%$ ($n = 5$) (Fig. 2A). By contrast, the rate of increase in the H₂ saturation was slower in the center of the thigh muscle with attaining a maximum level of $0.50 \pm 0.03\%$ ($n = 5$) after 30 min (Fig. 2B and Supplementary Fig.).

Of note, H₂ gas levels were increased even in the ischemic myocardium (Fig. 2C). Although the incremental rate of H₂ saturation was slower in the ischemic myocardium than in the non-ischemic myocardium, the peak level of H₂ in the ischemic myocardium was reached at approximately two thirds of the value observed in the

non-ischemic myocardium (Fig. 2D). After restoration of coronary artery blood flow, the level of H₂ in the ischemic myocardium immediately increased to the level observed in the non-ischemic myocardium.

Inhalation of H₂ gas protects the heart from ischemia–reperfusion injury

To investigate whether inhalation of H₂ gas protects the heart from ischemia–reperfusion injury, rats were subjected to coronary artery occlusion for 30 min followed by reperfusion for 24 h. H₂ gas was administered at the onset of ischemia and continued for 60 min after reperfusion. H₂ gas has no adverse effect on heart rate and arterial oxygenation (Fig. 3A). There was no significant difference in the temporal profile of LV end-systolic

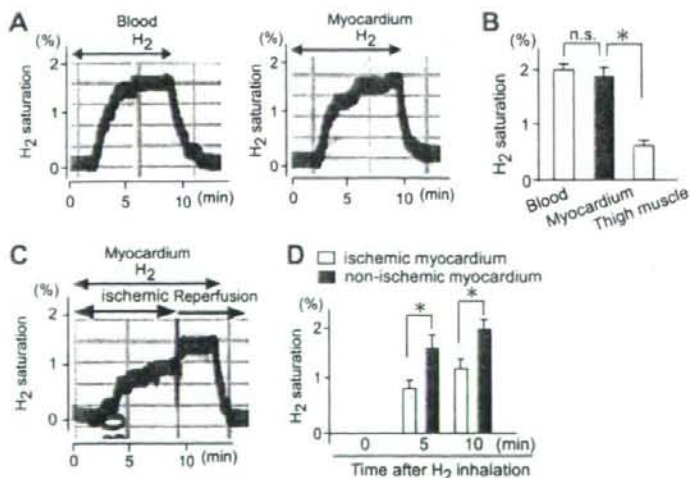


Fig. 2. Inhalation of H₂ gas increases the intramyocardial H₂ gas concentration. H₂ gas at 2% was administered by respiration to intubated rats receiving mechanical ventilation and the concentration of H₂ in tissue was recorded continuously. (A) A needle-type H₂ sensor was inserted in LV cavity (arterial blood) and non-ischemic LV myocardium. (B) Comparison of peak H₂ gas levels between arterial blood, non-ischemic LV myocardium, and thigh muscle ($n = 5$, $P < 0.05$, compared to the level of arterial blood). (C) The changes in the concentration of H₂ in 'at risk' area for infarction during ischemia and reperfusion. (D) Comparison of change in the H₂ concentration between non-ischemic and ischemic myocardium after H₂ inhalation ($n = 5$, $P < 0.05$, compared to the level of non-ischemic myocardium).

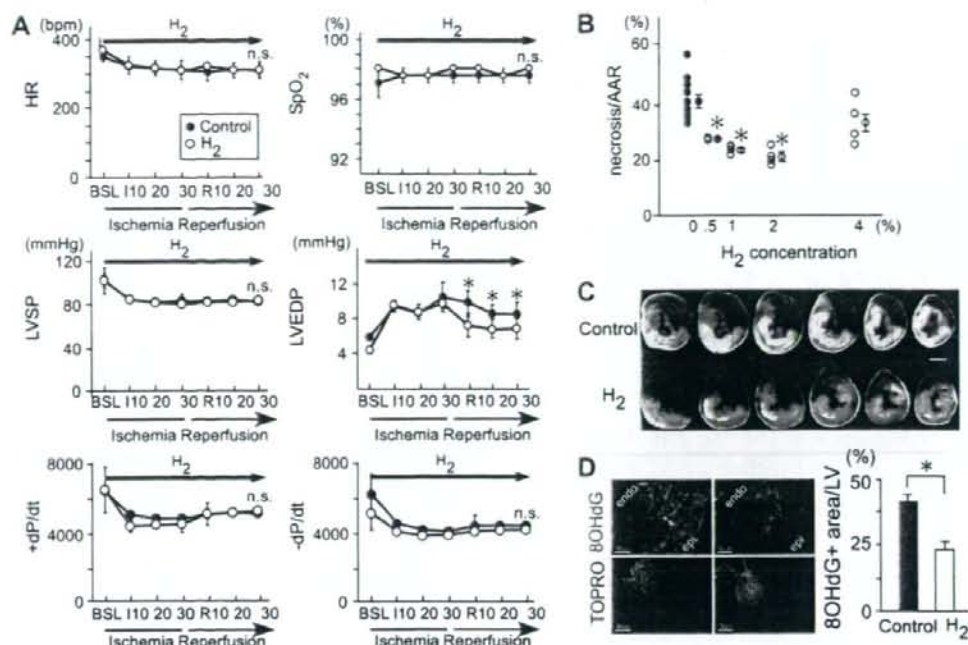


Fig. 3. Inhalation of H₂ reduces infarct size induced by ischemia–reperfusion injury. (A) Changes in heart rate (HR), oxygen saturation by pulse oximetry (SpO₂), and LV systolic pressure (LVSP), LV diastolic pressure (LVEDP) and LV peak positive and negative dP/dt were monitored during ischemia–reperfusion injury ($n = 5$ in each group). (B) H₂-dependent decrease in infarct size is expressed as the ratio of total infarct area/AAR ($P < 0.05$, compared to control group). (C) Representative photographs of serial heart sections obtained from rats subjected to myocardial ischemia–reperfusion injury in the presence or absence of H₂ inhalation. Bar = 2 mm. (D) Immunohistochemical staining with antibodies against 8-OHdG was performed 24 h after ischemia–reperfusion injury. Quantification of 8-OHdG immunoreactive area was expressed as percentage of total LV area at serial short axis sections ($n = 5$, $P < 0.05$, H₂ inhalation group compared to control group). endo, endocardium; epi, epicardium.

pressure, LV peak positive and negative LV dP/dt, between the control group and the 2% H₂ gas inhalation group. Notably, LV-end-diastolic pressure after reperfusion was significantly lower in H₂ gas inhalation group compared to control group ($n = 5$, $P < 0.05$).

In the absence of H₂ gas inhalation, infarct size following ischemia–reperfusion was $41.6 \pm 2.5\%$ of the area at risk ($n = 9$). By comparison, inhalation of 0.5–2% H₂ gas significantly reduced infarct size, with 2% H₂ gas providing the most prominent effects ($21.2 \pm 1.6\%$ of area at risk, $n = 4$, Fig. 3B and C). There was no significant difference in area at risk/LV among control group and H₂ gas inhalation groups (data not shown). Consistent with those observations, the quantitative determination of 8-hydroxydeoxyguanosine (8-OHdG) immunoreactive area, a biomarker of oxidative stress, revealed that the level of oxidative injury elicited in the 'at risk' area was significantly smaller in the group receiving 2% H₂ gas inhalation than that of control group ($n = 5$, $P < 0.05$, Fig. 3D).

Inhalation of H₂ gas reduces LV remodeling after ischemia–reperfusion injury

To determine the impact of H₂ inhalation at the time of ischemia–reperfusion on pathological LV remodeling, LV morphology and function were monitored by echocardiography 30 days after myocardial ischemia–reperfusion injury. Control rats showed maladaptive pathological remodeling after myocardial infarction, including dilatation of LV cavity, reduced LV systolic function. Notably, inhalation of H₂ gas during myocardial ischemia–reperfusion reduced pathological remodeling after myocardial infarction (Fig. 4).

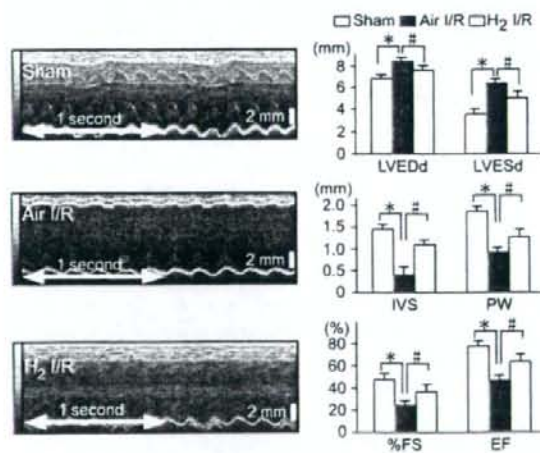


Fig. 4. Inhalation of H₂ gas reduces adverse LV remodeling. Representative M-mode echocardiographic images of sham-operated (sham), ischemia–reperfusion with air inhalation (Air_I/R), and ischemia–reperfusion with H₂ inhalation (H₂_I/R). Measurement of M-mode echocardiographic images in each group. LVEDd, LV end-diastolic diameter (μm); LVESd, LV end-systolic diameter (μm); IVS, intraventricular septum diameter (μm); PW, posterior wall thickness (μm); FS, fractional shortening (%); EF, ejection fraction (%) ($n = 5$, $P < 0.05$, compared to sham-operated group; $\#P < 0.05$, compared to Air_I/R group).

Discussion

This is the first study to demonstrate that inhalation of H₂ gas, at an incombustible level, limit the extent of myocardial infarction

resulting from myocardial ischemia-reperfusion injury, and thereby preserve LV function *in vivo*. The cardioprotective effect of H₂ is also confirmed *ex vivo* Langendorff-perfused hearts subjected to anoxia-reoxygenation injury. The anti-oxidant properties of H₂ were confirmed by the demonstration that (1) H₂ improves the recovery of LV function during reoxygenation after anoxia, (2) the oxidative stress model, in isolated perfused hearts; (3) inhalation of H₂ gas ameliorates the level of 8-OHdG immunoreactivity in the 'at risk' area for infarction. The anti-oxidant action of molecular H₂ may be explained, at least partially, by direct ROS scavenging effect. However, it remains unclear if the anti-oxidant action of H₂ is also ascribed to the activation of the reperfusion injury salvage kinase pathways or a direct effect on mitochondrial energetics.

Gas inhalation as disease therapy has received recent interest. There are three endogenous gas signaling molecules, known as gas transmitters, include nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S). The increased production of these gases under stress conditions may reflect the active involvement of these gases in the protective response. In pre-clinical experimental models of disease, including ischemia-reperfusion injury, the inhalation of exogenous CO or H₂S has produced a favorable outcome for most vital organs [19–22]. However, the inherent toxicity of these gases must be investigated for gas inhalation to be considered an effective therapeutic strategy. It is unknown if the therapeutically effective threshold for CO or H₂S can be attained locally in target organs without delivering a potentially toxic level of these gases via the lungs.

H₂ is not produced endogenously in mammalian cells since the hydrogenase activity responsible for the formation of H₂ has not been identified [23]. The spontaneous production of H₂ gas in the human body occurs via fermentation of undigested carbohydrates by resident enterobacterial flora. H₂ is transferred to the portal circulation and excreted through the breath in significant amounts. We demonstrated that inhaled H₂ at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (SpO₂) or hemodynamic parameters, including heart rate and LV pressure. H₂ dissolved in the blood is distributed to tissues proportional to regional blood flow, and is rapidly eliminated by the lungs. Accordingly, the H₂ gas clearance method was employed to measure local blood flow in various tissues [24]. Since the heart is one of the most highly perfused tissues, the intramyocardial H₂ concentration increases immediately following inhalation of H₂, and attaining to almost compatible levels of that observed in arterial blood within 3 min. Of note, the regional H₂ concentration in the ischemic myocardium reaches at two thirds of the value observed in the non-ischemic myocardium. This may occur through gaseous diffusion from the blood in the ventricular cavity and/or adjacent non-ischemic myocardium. These findings indicate that administration of H₂ gas by inhalation, in patients with totally coronary artery occlusion, can efficiently increase the regional concentration of H₂ in the 'at risk' area for myocardial infarction before reestablishing coronary blood flow within the occluded infarcted artery.

We demonstrated that inhalation of H₂ gas is promising strategies to alleviate ischemia-reperfusion injury at the time of recanalization of coronary artery. When translated into the clinical practice, inhalation of H₂ gas must be most frequently applied in the treatment of patients with acute myocardial infarction in conjunction with routinely performed PCI procedures. Further understanding of the mechanisms underlying the signaling pathways involved in H₂-mediated anti-oxidant activity, and the capacity of H₂ to influence cellular metabolism, are required to fully exploit inhalation of H₂ gas as a therapeutic strategy.

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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.2008.05.165.

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Letter to the Editor

Is mitral regurgitant jet offensive rather than protective for left atrial thrombus?

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Keywords: Left atrial thrombus; Mitral regurgitant jet; Atrial fibrillation; Malignancy; Anticoagulant therapy

A 77-year-old man with chronic atrial fibrillation (AF) was admitted to our hospital for dysarthria. Diffusion magnetic resonance imaging showed multiple high signals in the brain, indicating acute emboli. Trans-esophageal echocardiography revealed a giant thrombus in the left atrial (LA) roof with mobile process on its surface. Surprisingly, it was also observed that the surface of thrombus was encroached on by mitral regurgitant (MR) jet (Fig. 1). No pericardial effusion and extra cardiac mass were observed. No remarkable coagulation factor abnormality was detected. Open chest thrombectomy concomitant with simple isolation of pulmonary vein (PV) orifices for AF was successfully performed to prevent a recurrence of lethal emboli. The pathological examination revealed no other contents like cancer cells in the extracted thrombus. He recovered uneventfully after the operation and not only thrombus but also AF disappeared. Oral anticoagulation

was started, and then he was discharged. Two months after discharge, he was re-admitted for consciousness disturbance due to hypercalcemia (12.9 mg/dl, corrected). Computed tomography, tumor markers (SCC 3.1 ng/ml, PTH-rp 7.1 pmol/ml) and trans-bronchial lung biopsy indicated that he had a squamous cell carcinoma in the left lower lung lobe. No obvious direct cardiac invasion of carcinoma was documented. Although warfarin was effected (PT-INR 3.4) and moderate MR jet was blown under the recurrence of AF, a giant thrombus reappeared in the LA roof (Fig. 2). No other systemic thrombus was detected except for the LA. Because of metastasis, he was taken the best supportive therapy, and the thrombus was slowly progressive in the LA until he died.

AF is a familiar arrhythmia and is an independent risk factor for emboli. Oral anticoagulation markedly decreases the risk of emboli in patients with AF [1]. It was also reported that the presence of significant MR correlates with a lower incidence of emboli [2]. On the other hand, strong clinical evidence is accumulating on the hypercoagulability of cancer patients [3].

Although we couldn't clearly explain why the thrombus reappeared on the same place, we could speculate that the thrombus was mainly formed at the PV because the concentration of thrombophilic products from lung cancer

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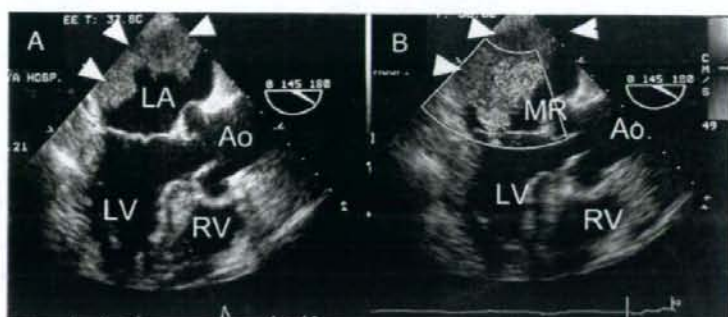


Fig. 1. Trans-esophageal echocardiograms showing a giant thrombus (arrow heads) in the left atrium at the first admission. (A) Moderate mitral regurgitant jet blowing and encroaching on the surface of the thrombus. (B) LV indicates left ventricle; RV, right ventricle; LA, left atrium; Ao, aorta.

cells are higher at the PV, where the blood flow was reduced due to AF. To our knowledge, this is the first description about a recurrent LA giant thrombus regardless of MR jet and anticoagulant therapy in a 77-year-old man with AF. Clinicians should note the potentially offensiveness of MR jet in the AF patients with malignancy, which could overcome to form a thrombus against all defenses.

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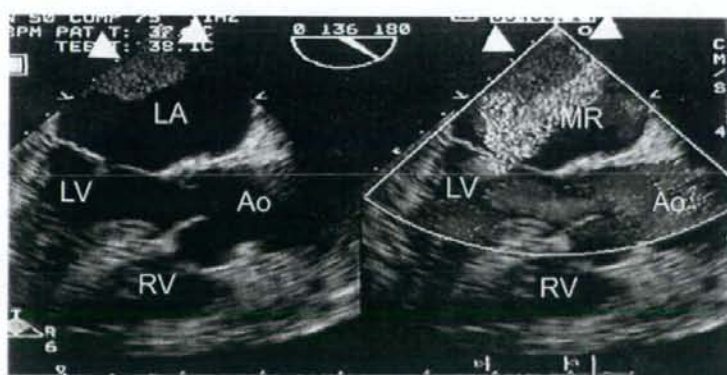


Fig. 2. Trans-esophageal echocardiograms showing a recurrent giant thrombus (arrow heads) in the left atrium at the second admission regardless of severe mitral regurgitant jet. MR indicates mitral regurgitation.

Regulation of Cardiac Nerves: A New Paradigm in The Management of Sudden Cardiac Death?

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Abstract: The heart is extensively innervated, and its performance is tightly regulated by the autonomic nervous system. To maintain cardiac function, innervation density is stringently controlled, being high in the subepicardium and the central conduction system. In diseased hearts, cardiac innervation density varies, which in turn leads to sudden cardiac death. After myocardial infarction, sympathetic denervation is followed by reinnervation within the heart, leading to unbalanced neural activation and lethal arrhythmia. Diabetic sensory neuropathy causes silent myocardial ischemia, characterized by loss of pain perception during myocardial ischemia, which is a major cause of sudden cardiac death in diabetes mellitus (DM). Despite its clinical importance, the molecular mechanism underlying innervation density remains poorly understood.

We found that cardiac sympathetic innervation is determined by the balance between neural chemoattraction and chemorepulsion, both of which occur in the heart. Nerve growth factor (NGF), which is a potent chemoattractant, is synthesized abundantly by cardiomyocytes and is induced by endothelin-1 upregulation in the heart. In contrast, *Sema3a*, which is a neural chemorepellent, is expressed strongly in the trabecular layer in early stage embryos and at a lower level after birth, leading to epicardial-to-endocardial transmural sympathetic innervation patterning. We also found that cardiac NGF downregulation is a cause of diabetic neuropathy, and that NGF supplementation rescues silent myocardial ischemia in DM. Both *Sema3a*-deficient and *Sema3a*-overexpressing mice showed sudden death or lethal arrhythmias due to disruption of innervation patterning. The present review focuses on the regulatory mechanisms involved in neural development in the heart and their critical roles in cardiac performance.

Keywords: Heart, cardiac nerve, nerve growth factor, *Sema3a*, arrhythmia, sudden cardiac death.

INTRODUCTION

Cardiac tissues are extensively innervated by autonomic nerves. The sympathetic nervous system produces norepinephrine and increases the heart rate, conduction velocity, as well as myocardial contraction and relaxation. It is well known that sympathetic innervation density, which is high in the subepicardium and the central conduction system, is determined stringently within the heart [1-4]. The regional difference in sympathetic innervation influences cardiac functions to different extents, effectively controlling the heart rate and myocardial contraction and relaxation. Despite its importance, little is known about the developmental and regulatory mechanisms underlying cardiac sympathetic innervation patterning. Moreover, to date there has been no experimental demonstration of the consequences of disrupting this patterning.

Cardiac innervation density is altered in diseased hearts, as in cases of congestive heart failure and myocardial infarction [5-7]. Following myocardial injury, cardiac nerves undergo Wallerian degeneration, which may be followed by neurilemmal cell proliferation and axonal regeneration, resulting in heterogeneous innervation [8, 9]. Unbalanced sympathetic innervation may trigger lethal arrhythmia through ion channel modulation in cardiomyocytes [7, 10, 11]. On the other hand, as the sensory nervous system is responsible for pain perception, cardiac sensory denervation may cause silent myocardial ischemia, characterized by loss of pain perception during myocardial ischemia and frequently leading to sudden cardiac death (SCD) in diabetes mellitus (DM) patients [12]. Despite the severity of these complications, the molecular mechanism that determines innervation density in pathological hearts is poorly understood. Addressing these questions requires a better understanding of the anatomical distribution of cardiac nerves and the molecular mechanism that regulates innervation during development.

In this article, we review the regulatory mechanisms involved in neural development in the heart, as well as its critical roles in cardiac performance.

CARDIAC NERVE DEVELOPMENT

Neural crest cells migrate and form sympathetic ganglia by mid-gestation, and subsequently proliferate and differentiate into mature neurons [13, 14]. The cardiac sympathetic nerves extend from the sympathetic neurons in stellate ganglia, which are located bilateral to the vertebra. Sympathetic nerve fibers project from the base of the heart into the myocardium, and are located predominantly in the subepicardium of the ventricle [1, 2]. The central conduction system, which includes the sinoatrial node, atrioventricular node, and His bundle, is abundantly innervated compared with the working myocardium [2, 4, 15, 16]. We and others have reported that this regional difference in cardiac sympathetic innervation (innervation patterning) is highly conserved among mammals [2, 4, 17].

The cardiac nervous system also involves afferent nerves. The sensory signals generated in the heart are conducted through cardiac afferent nerves, primarily thinly myelinated A δ -fibers and nonmyelinated C-fibers [18, 19]. The sensory nerve fibers project to the upper thoracic dorsal horn via dorsal root ganglia neurons, which are also derived from neural crest cells [18, 19].

One of the difficulties in analyzing cardiac innervation of the heart has been the lack of suitable molecular markers. However, recent advancements in immunohistochemical techniques have allowed us to stain autonomic nerves using antibodies against nerve-specific markers, such as tyrosine hydroxylase (TH; a sympathetic marker), calcitonin gene-related peptide (CGRP; a sensory marker), protein gene product 9.5 (PGP 9.5; a general peripheral nerve marker), and growth associated protein 43 (GAP43; a nerve sprouting marker). As discussed below, using these specific neural markers, we and others have recently demonstrated that cardiac innervation is strictly organized in the heart during development, whereas in diseased hearts, innervation density appears to be altered dynamically [5, 9, 20-22].

NERVE SPROUTING AND SUDDEN CARDIAC DEATH

It is well known that sympathetic stimulation is important in the generation of SCD in diseased hearts. There is a circadian variation of the frequency of SCD in parallel with sympathetic activity. β -

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Blocker therapy prevents SCD secondary to ventricular tachyarrhythmia in ischemic heart disease or congestive heart failure [23, 24]. Immunohistochemical analysis of cardiac nerves in explanted hearts of transplant recipients has revealed a positive correlation between nerve density and clinical history of ventricular tachyarrhythmia [7]. Zhou *et al.* [25] have shown that nerve growth factor (NGF), which is critical for sympathetic nerve sprouting, is upregulated after myocardial infarction (MI) in animal models, resulting in the regeneration of cardiac sympathetic nerves and heterogeneous innervation. In other experiments, it has been shown that augmented myocardial nerve sprouting through NGF infusion after MI results in a dramatic increase in SCD and a high incidence of ventricular tachyarrhythmia, as compared with animals not receiving NGF infusion [5]. These results demonstrate that NGF upregulation and nerve sprouting in diseased hearts may cause lethal arrhythmia and SCD. Despite its importance, the molecular mechanisms that regulate NGF expression and sympathetic innervation in the heart are poorly understood.

THE ENDOTHELIN-1/NGF PATHWAY IS CRITICAL FOR CARDIAC SYMPATHETIC INNERVATION

In general, the growth-cone behavior of nerves is modulated by coincident signaling modulated by neural chemoattractants and chemorepellents synthesized in the innervated tissue. NGF, which is a potent neural chemoattractant, is a prototypic member of the neurotrophin family, the members of which are critical for the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems [26-28]. The levels of NGF expression within innervated tissues correspond approximately to the levels of innervation density [29]. NGF expression increases during development and is altered in diseased hearts [5-7, 30]. In spite of the importance of NGF in neural development, the upstream molecules that regulate NGF expression *in vivo* remained undetermined [31].

Endothelin-1 (ET-1) is a critical factor in the pathogenesis of cardiac hypertrophy, hypertension, and atherosclerosis [32, 33]. Gene targeting of ET-1 and its receptor ET_A has resulted in unexpected craniofacial and cardiovascular abnormalities that have not been observed in other hypertrophic factor-deficient mice [34-37]. These phenotypes are consistent with interference of neural crest differentiation. However, the influence of ET-1 on neural crest development remains to be determined. Therefore, we hypothesized that ET-1 might affect the induction of neurotrophic factors, and that its disruption might contribute to the immature development of neural crest-derived cells [20].

We found that ET-1 (among several hypertrophic factors tested) specifically upregulated NGF expression in primary cultured cardiomyocytes. ET-1-induced NGF augmentation was not observed in cardiac fibroblasts, and this phenomenon was cell type-specific in cardiomyocytes. We also identified the signaling molecules involved in the ET-1/NGF pathway [38]. To study the effects of the ET-1/NGF pathway on the development of the cardiac sympathetic nervous system, we analyzed various gene-modified mouse models. NGF expression, cardiac sympathetic innervation (Fig. 1), and norepinephrine concentration were reduced in ET-1-deficient mouse (*Edn1*^{-/-}) hearts, but not in the hearts of angiotensinogen-deficient mice (*Atg*^{-/-}). In *Edn1*^{-/-} mice, the sympathetic stellate ganglia exhibited excessive apoptosis and displayed loss of neurons at the late embryonic stage [14]. Moreover, we have demonstrated that cardiac-specific overexpression of NGF in *Edn1*^{-/-} mice overcomes sympathetic nerve retardation [39]. These findings indicate that ET-1 is a key regulator of NGF expression in cardiomyocytes, and that the ET-1/NGF pathway is critical for sympathetic innervation in the heart [20].

We have previously investigated whether the ET-1/NGF pathway plays a role in sympathetic innervation in diseased hearts [40]. We produced right ventricular hypertrophy in rats by monocrotaline treatment, and analyzed the levels of NGF expression and newly developed sympathetic nerves in the hearts [41, 42]. The levels of ET-1, NGF, and brain natriuretic peptide (hypertrophy marker) expression were strongly increased in the right ventricles in monocrotaline-treated rats [43-45]. Sympathetic nerves that were immunopositive for polysialylated neural cell adhesion molecule (PSA-NCAM; an immature neuron marker), β 3-tubulin (axonal marker) and GAP 43, were markedly increased in the hypertrophied right ventricles, in parallel with NGF upregulation. Thus, the ET-1/NGF pathway contributes to anatomical sympathetic hyperinnervation in pressure overload-induced cardiac hypertrophy. Given that ET-1 is strongly induced in myocardial infarction, the ET-1/NGF pathway may also be involved in NGF upregulation and nerve regeneration after myocardial infarction.

NERVE GROWTH FACTOR IS CRITICAL FOR CARDIAC SENSORY INNERVATION AND RESCUES NEUROPATHY IN DIABETIC HEARTS

The cardiac autonomic nervous system is composed of efferent and afferent nerves. The cardiac sensory nervous system is responsible for pain perception and for initiating a protective cardiovascular response during myocardial ischemia [18, 19, 46, 47]. Cardiac sensory nerve impairment causes silent myocardial ischemia, which

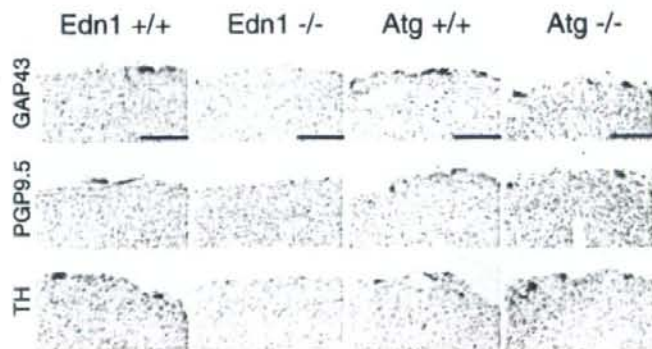


Fig. (1). Disruption of ET-1, but not of angiotensinogen, reduces sympathetic nerve density in murine hearts.

Immunostaining for GAP43, PGP9.5, and TH in the heart. Note that GAP43-, PGP9.5-, and TH-immunopositive nerves are detected less frequently in *Edn1*^{-/-} mice, but not in *Atg*^{-/-} mice, as compared to the respective WT littermates. Scale bar, 100 μ m.

is a major cause of sudden death in DM patients [12]. Despite the severity of this complication, the alterations in cardiac sensory innervation and the molecular mechanism that underlies sensory neuropathy in diabetic hearts are poorly understood [48-53]. Moreover, little is known about the anatomical distribution of cardiac sensory nerves and the molecular mechanism of innervation during development [54].

Unlike somatic tissues, visceral organs, such as the heart, are believed to be rich in autonomic efferent innervation but poor in nociceptive afferent nerves [55]. In fact, Zahner *et al.* [56] have reported that vanilloid receptor-1-immunopositive sensory nerves are enriched in the epicardium but scarce in the myocardium. We have identified for the first time that cardiac sensory innervation is rich not only at epicardial sites but also in the ventricular myocardium, and that sensory innervation increases with development [21, 57]. In our screen of several neurotrophic factors, we have found that cardiac sensory nerves develop in parallel with NGF synthesized in the heart [58]. Cardiac nociceptive sensory nerves that are immunopositive for CGRP, the dorsal root ganglia, and the dorsal horn are markedly retarded in NGF-deficient mice, while cardiac-specific overexpression of NGF rescues these deficits. Thus, NGF synthesis in the heart is critical for the development of the cardiac sensory nervous system [59].

To investigate whether NGF is involved in diabetic neuropathy, DM was induced with streptozotocin in wild-type (WT) and transgenic mice that overexpressed NGF in the heart [21, 60-63]. Downregulation of NGF, CGRP-immunopositive cardiac sensory denervation, and atrophic changes in dorsal root ganglia were observed in DM-induced WT mice, whereas these deteriorations were rescued in DM-induced NGF-transgenic mice (Fig. 2). Cardiac sensory function, as measured by myocardial ischemia-induced c-Fos expression in dorsal root ganglia, was also downregulated by DM in the WT mice, but not in the NGF-transgenic mice [19]. Direct gene transfer of NGF into the diabetic rat hearts improved the impaired cardiac sensory innervation and function, as determined by the electrophysiological activities of cardiac afferent nerves during myocardial ischemia [64, 65]. These findings demonstrate that the development of the cardiac sensory nervous system is dependent on NGF synthesized in the heart, and that DM-induced NGF reduction may lead to cardiac sensory neuropathy.

Phase I and phase II clinical trials of systemic administration of recombinant NGF have revealed safety and potential efficacy in diabetic polyneuropathy, although a phase III trial did not show beneficial effects, as the dosage and route of administration may have been suboptimal [66, 67]. The dosage of NGF was restricted

by side-effects, and the development of anti-NGF antibodies may have contributed to the lack of beneficial effects in the phase III clinical trial. These complications might be avoided by directly administering the NGF gene to the cells that require the factor. NGF- and CGRP-immunopositive nerves were proportionally reduced in diabetic hearts, and thus we successfully treated cardiac sensory neuropathy by direct NGF gene transfer. Consistent with our findings, the efficacy of NGF gene therapy has been reported in diabetic cystopathy and neuropathy of the footpad [67, 68]. Further studies on the reliability and efficacy of this gene therapy are required before clinical trials can proceed.

SEMA3A IS CRITICAL FOR CARDIAC SYMPATHETIC INNERVATION PATTERNING

As discussed above, NGF plays critical roles in cardiac nerve development. In contrast, the neural chemorepellent that induces growth-cone collapse and repels nerve axons has not been identified in the heart. *Sema3a*, which is a Class 3 secreted semaphorin, has been cloned and identified as a potent neural chemorepellent and a directional guidance molecule for nerve fibers [69-71]. However, it is not known whether cardiomyocytes produce *Sema3a*, and if so, whether this protein affects sympathetic neural patterning and cardiac performance.

We analyzed the kinetics and distribution of cardiac sympathetic innervation in developing murine ventricles [22]. TH-immunopositive sympathetic nerve endings appeared on the epicardial surface at embryonic day (E)15, and gradually increased in number in the myocardium after postnatal day (P)7 and P42. In the ventricular myocardium, sympathetic nerves were found to be more abundant in the subepicardium than in the subendocardium, suggesting an epicardial-to-endocardial gradient [1, 4, 15, 16]. To identify the *Sema3a* expression pattern and its relationship to innervation patterning in the heart, we analyzed heterozygous *Sema3a* knocked-in *lacZ* mice (*Sema3a^{lacZ}*). At E12, *lacZ* expression was detected strongly in the heart, especially in the trabecular components of the ventricles. In E15 hearts, *lacZ* expression was observed in the subendocardium but not in the subepicardium of the atria and ventricles. At P1 and P42, *lacZ* expression was reduced in certain regions and highlighted the Purkinje fiber network along the ventricular free wall [72, 73]. Quantitative RT-PCR of *Sema3a* in developing hearts also revealed the presence of *Sema3a* from E12, with subsequent linear decrease in expression, in contrast to the pattern of sympathetic innervation. These results indicate that *Sema3a* shows the opposite kinetics and distribution pattern of expression to the pattern of sympathetic innervation in developing

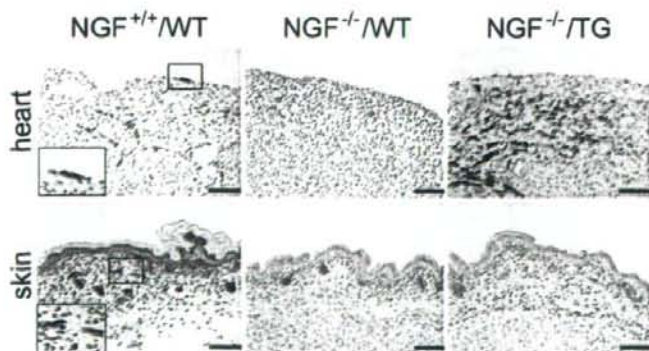


Fig. (2). Cardiac-specific overexpression of NGF rescues the cardiac sensory nervous system defects in NGF-deficient mice.

Immunostaining for CGRP in the hearts and limb skin of *NGF^{+/+}/WT*, *NGF^{-/-}/WT*, and *NGF^{-/-}/TG* mice. Note that *NGF^{-/-}/TG* hearts are hyperinnervated compared to *NGF^{+/+}/WT* littermates. In contrast, there are no differences in skin denervation. Scale bar, 100 μ m.

hearts, which suggests that *Sema3a* negatively regulates cardiac innervation (Fig. 3).

To investigate whether *Sema3a* is critical for cardiac sympathetic nerve development, we analyzed *Sema3a*-deficient mice (*Sema3a*^{-/-}) [74, 75]. The WT hearts showed a clear epicardial-to-endocardial gradient of sympathetic innervation. In contrast, the sympathetic nerve density was reduced in the subepicardium but increased in the subendocardium of *Sema3a*^{-/-} mice, resulting in disruption of the innervation gradient in *Sema3a*^{-/-} ventricles. The *Sema3a*^{-/-} mice also exhibited malformation of the stellate ganglia that extend sympathetic nerves to the heart. To investigate whether the abnormal sympathetic innervation patterning in *Sema3a*^{-/-} hearts is a secondary effect of stellate ganglia malformation, we generated transgenic mice that overexpressed *Sema3a* specifically in the heart (*SemaTG*) [76]. *SemaTG* mice were associated with reduced sympathetic innervation and attenuation of the epicardial-to-endocardial innervation gradient. These results indicate that cardiomyocyte-derived *Sema3a* plays critical roles in cardiac sympathetic innervation by inhibiting neural growth. Since cardiomyocyte-derived NGF acts as a chemoattractant, it is possible that the balance between NGF and *Sema3a* synthesized in the heart determines cardiac sympathetic innervation patterning.

The growth-cone behavior of somatic sensory axons is also modulated by coincident signaling between NGF and *Sema3a* [77, 78]. During development, NGF and *Sema3a* are expressed within the spinal cord and influence the pathway guidance of sensory axons. *Sema3a* is specifically expressed in the ventral half of the spinal cord and mediates NGF-responsive sensory axons to terminate at the dorsal part of the spinal cord [69, 79]. Targeted inactivation of *Sema3a* disrupts neural patterning and projections in the spinal cord, which underscores the importance of *Sema3a* signaling for the directional guidance of nerve fibers [74, 75].

SEMA3A MAINTAINS ARRHYTHMIA-FREE HEARTS THROUGH SYMPATHETIC INNERVATION PATTERNING

Most of the *Sema3a*^{-/-} mice died within the first postnatal week, with only 20% surviving viable until weaning [74, 75]. To identify the cause of death and the effects of abnormal sympathetic neural distribution in *Sema3a*^{-/-} hearts, we performed telemetric electro-

cardiography and heart-rate variability analysis [80, 81]. In addition to multiple premature ventricular contractions, *Sema3a*^{-/-} mice developed sinus bradycardia and abrupt sinus arrest due to sympathetic neural dysfunction.

The *SemaTG* mice died suddenly without any symptoms at 10 months of age. Sustained ventricular tachyarrhythmia was induced in *SemaTG* mice but not in WT mice after epinephrine administration, and programmed electrical stimulation revealed that *SemaTG* mice were highly susceptible to ventricular tachyarrhythmia [82, 83]. The β -adrenergic receptor density was upregulated and the cAMP response after catecholamine injection was exaggerated in *SemaTG* ventricles. Action potential duration was significantly prolonged in hypoinnervated *SemaTG* ventricles, presumably via ion channel modulation. These results suggest that the higher susceptibility of *SemaTG* mice to ventricular arrhythmia is due at least in part to catecholamine supersensitivity and prolongation of action potential duration, both of which can augment triggered activity in cardiomyocytes [84-88]. Thus, *Sema3a*-mediated sympathetic innervation patterning is critical for the maintenance of arrhythmia-free hearts.

Sympathetic nerves modulate the function of ion channels and trigger various arrhythmias in diseased hearts [10, 11]. However, the relationship between sympathetic innervation and arrhythmogenicity in structurally normal hearts has been unclear. Induction of sympathetic hyperinnervation in normal adult canine hearts by either NGF infusion or subthreshold electrical stimulation of the left stellate ganglion resulted in no arrhythmia except for sinus tachycardia. However, the same magnitude of sympathetic hyperinnervation in diseased hearts cause increased incidence and sudden death [5]. These findings suggest that sympathetic hyperinnervation in normal canine hearts are not arrhythmogenic. On the other hand, the *Sema3a*^{-/-} mice exhibited sinus bradycardia, abrupt sinus slowing, and stellate ganglia defects. Consistent with our data, right stellectomy has been shown to induce sinus bradycardia and sudden, asystolic death in dogs [89]. In addition, Stramba-Badiale *et al.* [90] have reported that a developmental abnormality in cardiac innervation may play a role in the genesis of some cases of sudden infant death syndrome. Thus, the onset and duration of sympathetic neural dysfunction might be critical for arrhythmogenicity in structurally normal hearts. The *SemaTG* hearts were also highly susceptible to ventricular arrhythmias, albeit without contractile dysfunction.

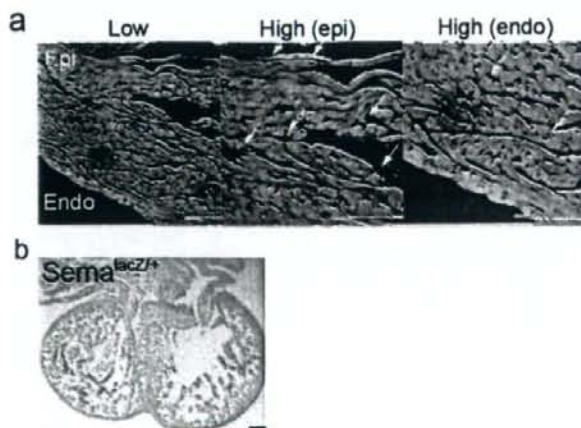


Fig. (3). Inverse expression pattern of *Sema3a* and sympathetic innervation in murine hearts.

(a) Immunofluorescence staining for α -actinin (red), TH (green), and TOTO-3 (blue, nuclei). TH-immunopositive nerves (arrows) are more abundant in the subepicardium (Epi) than in the subendocardium (Endo). Low and High indicate low-power and high-power magnification fields, respectively. (b) X-gal staining (green) of *Sema3a*^{lacZ/+} hearts at E12 demonstrates strong *Sema3a* expression in the subendocardium. Scale bar, 100 μ m.

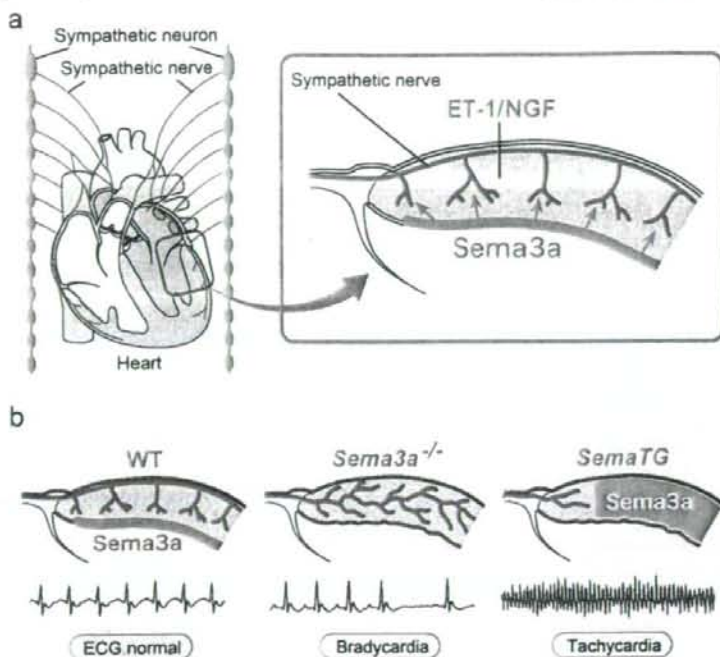


Fig. (4). Regulation of cardiac innervation patterning. (a) Cardiac sympathetic innervation shows an epicardial-to-endocardial transmural gradient. This patterning is established by the balance between ET-1/NGF and Sema3a expression in the heart. Note that NGF is expressed abundantly in the working myocardium, whereas Sema3a is expressed specifically in the subendocardium. (b) Appropriate Sema3a-mediated sympathetic innervation patterning is critical for the maintenance of an arrhythmia-free heart. *Sema3a*^{-/-} mice exhibit sinus bradycardia, and *SemaTG* mice are highly susceptible to ventricular tachyarrhythmias.

tion or structural defects. Given that catecholamine augments systolic function, it is surprising that the *SemaTG* mice showed normal cardiac function. However, patients who underwent heart transplantation and had denervated hearts did not show heart failure, while approximately 10% of the patients developed SCD [91]. These results suggest that the regulation of cardiac nerves should be a new paradigm for the management of SCD.

CONCLUSIONS

Cardiac nerves are highly plastic, and innervation patterning is strictly controlled by the balance between NGF and Sema3a synthesized in the heart (Fig. 4a). ET-1 regulates NGF expression in cardiomyocytes, and the ET-1/NGF pathway modulates nerve sprouting and plays critical roles in sympathetic nerve development [20, 40]. NGF is also important in sensory nerve development, and NGF downregulation may result in sensory neuropathy in diabetic hearts [21]. On the other hand, Sema3a inhibits neural growth and establishes appropriate innervation patterning in the heart. The disruption of sympathetic innervation patterning may lead to SCD, not only in diseased hearts but also in developing hearts (Fig. 4b) [22]. Knowledge of the mechanisms regulating cardiac innervation patterning in hearts represents an important step towards the development of therapies for SCD.

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Tissue- and Context-Dependent Modulation of Hormonal Sensitivity of Glucocorticoid-Responsive Genes by Hexamethylene Bisacetamide-Inducible Protein 1

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Physiological and pharmacological processes mediated by glucocorticoids involve tissue- and context-specific regulation of glucocorticoid-responsive gene expression via glucocorticoid receptor (GR). However, the molecular mechanisms underlying such highly coordinated regulation of glucocorticoid actions remain to be studied. We here addressed this issue using *atp1a1* and *scnn1a*, both of which are up-regulated in response to corticosteroids in human embryonic kidney-derived 293 cells, but resistant in liver-derived HepG2 cells. Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) represses gene expression via, at least, two distinct mechanisms, i.e. positive transcription elongation factor b sequestration and direct interaction with GR, and is relatively high in HepG2 cells compared with 293 cells. Given this, we focused on the role of HEXIM1 in transcriptional regulation of these GR target genes. In HepG2 cells, hormone re-

sistance of *atp1a1* and *scnn1a* was diminished by either knockdown of HEXIM1 or overexpression of GR. Such a positive effect of exogenous expression of GR was counteracted by concomitant overexpression of HEXIM1, indicating the balance between GR and HEXIM1 modulates hormonal sensitivity of these genes. In support of this, the hormone-dependent recruitment of RNA polymerase II onto *atp1a1* promoter was in parallel with that of GR. Moreover, we revealed that not positive transcription elongation factor b-suppressing activity but direct interaction with GR of HEXIM1 plays a major role in suppression of promoter recruitment of the receptor and subsequent *atp1a1* and *scnn1a* gene activation. Collectively, we may conclude that HEXIM1 may participate in tissue-selective determination of glucocorticoid sensitivity via direct interaction with GR at least in certain gene sets including *atp1a1* and *scnn1a*. (*Molecular Endocrinology* 22: 2609–2623, 2008)

GLUCOCORTICOIDS ARE secreted from the adrenal glands under the strict control of the hypothalamic-pituitary-adrenal axis and maintain

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Abbreviations: AhR, Arylhydrocarbon receptor; BR, basic region; CDK9, cyclin-dependent kinase 9; ChIP, chromatin immunoprecipitation; CycT1, cyclin T1; DEX, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HA, hemagglutinin; HSF, heat shock factor; 3MC, 3-methylcholanthrene; MOI, multiplicity of infection; PPAR, peroxisome proliferator-activated receptor; PPARRE, PPAR response element; qRT-PCR, quantitative real-time RT-PCR; P-TEFb, positive transcription elongation factor b; RNAPII, RNA polymerase II; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; snRNA, small nuclear RNA; SR, siRNA-resistant; STAT3, signal transducer and activator of transcription 3; SV, simian virus; TGZ, troglitazone; TSA, trichostatin A.

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homeostasis through the regulation of electrolyte balance, glucose homeostasis, lipid and protein metabolism, and modulation of the immune, cardiovascular, and central nervous system (1–3). On the other hand, glucocorticoids have been widely and successfully used in treating a number of pathological states, e.g. inflammation and autoimmune disorders (4). It has been demonstrated that such physiological and pharmacological processes mediated by glucocorticoids involve tissue-specific regulation of glucocorticoid-responsive gene expression (3). Moreover, glucocorticoid sensitivity of every single gene has been shown to differ among cells, tissues, and individuals, and even fluctuates not only in pathological states, but also during normal physiological processes, including development and the cell cycle (4, 5). Albeit its importance, the molecular mechanisms underlying highly coordinated tissue- and context-dependent regulation of expression of glucocorticoid-target genes remain to be studied.

atp1a1 is expressed in all mammalian cells, and its product Na⁺, K⁺-ATPase α 1 plays essential roles in regulating ionic intracellular milieu, the process that is needed for the regulation of metabolism, proliferation, differentiation, and cell volume (6). In addition, in kidney, Na⁺, K⁺-ATPase α 1 also plays a central role in the fine control of systemic electrolyte balance through hormone-regulated sodium reabsorption, in functional cooperation with amiloride-sensitive Na⁺ channel, which is encoded by *scnn1a* (7–9). *atp1a1*, as well as *scnn1a*, is up-regulated in response to corticosteroids in kidney (7). On the other hand, particularly in liver, expression of either *atp1a1* or *scnn1a* is not influenced by glucocorticoids, indicating that these genes are resistant to glucocorticoids, not in kidney but in liver. Interestingly, their hormone resistance seems to be corticosteroid signal selective, because other extracellular stimuli, such as thyroid hormones (10) and low external potassium ion (11), were shown to modulate mRNA expression of *atp1a1* even in liver.

Glucocorticoids elicit their hormone actions via a signal pathway involving ubiquitously expressed glucocorticoid receptor (GR), a prototypic member of the nuclear receptor superfamily, which acts as a ligand-dependent transcription factor (12). It is generally believed that, upon binding glucocorticoids, GR translocates into the nucleus and binds the glucocorticoid response element (GRE) on the target gene promoters. Binding of ligand receptors with target DNA is followed by recruitment of mediators and coactivators to the proximity of the target DNA, resulting in RNA polymerase II (RNAPII) recruitment and activation of transcription (4, 13–19). The recent advent of DNA microarray technology has revealed that there are only modest overlaps in glucocorticoid-regulated gene sets among different cell types. In fact, considerable numbers of genes are responsive to glucocorticoids in certain tissues but resistant in others (20–23). Already several mechanisms have been postulated for tissue-specific regulation of glucocorticoid actions including different metabolisms of ligands (24), tissue-specific cofactor availability (25), and GR subtype distribution (26).

Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) was originally identified as a nuclear protein, expression of which was induced when human vascular smooth muscle cells were treated with hexamethylene bisacetamide, an inhibitor of cell proliferation (27). HEXIM1 has been shown to regulate mRNA expression via, at least, two distinct mechanisms, *i.e.* positive transcription elongation factor b (P-TEFb)-dependent (28, 29) and P-TEFb-independent mechanisms (30). P-TEFb, typically composed of cyclin-dependent kinase 9 (CDK9) and its regulatory partner cyclin T1 (CycT1), phosphorylates the C-terminal domain of RNAPII, thereby stimulating transcription elongation (31–33). P-TEFb recruitment has been reported in diverse class II promoters in association with a certain class of transcription factors, including HIV-1

Tat (34), nuclear factor- κ B (35), signal transducer and activator of transcription 3 (STAT3) (36), heat shock factor (HSF) (37), and arylhydrocarbon receptor (AhR) (38). HEXIM1 exerts its inhibitory effect on P-TEFb *in vivo* and *in vitro* in a 7SK small nuclear RNA (snRNA)-dependent fashion (28, 29). Upon binding with HEXIM1 and 7SK snRNA, P-TEFb loses its kinase activity, resulting in suppression of transcription elongation (39, 40). On the other hand, several reports described P-TEFb-independent mechanisms of gene regulation by HEXIM1 (30, 41–47). Among others, we demonstrated that HEXIM1 directly interacts with GR and modulates glucocorticoid-responsive gene expression (30). Moreover, we recently showed that GR, via its hinge region, interacts with central basic amino acid-rich region of HEXIM1 (48). At this moment, however, it remains unknown how genes can differentially use these distinct functions of HEXIM1: inhibitory effects on P-TEFb-dependent elongation and GR-mediated transactivation.

In the present study, we showed that mRNA expression of *atp1a1* and *scnn1a* was up-regulated by treatment with glucocorticoids in human embryonic kidney-derived 293 cells, but not in human liver cancer-derived HepG2 cells. Knockdown of endogenous HEXIM1 in HepG2 cells canceled glucocorticoid resistance of *atp1a1* and *scnn1a* mRNA expression. By creating a system that enables differential analysis of the above-mentioned distinct HEXIM1 functions, we revealed that not P-TEFb-suppressing activity but direct interaction with GR plays a major role in suppression of *atp1a1* activation by attenuating promoter recruitment of the receptor and RNAPII. We may conclude, therefore, that HEXIM1 may participate in tissue- and gene-selective determination of glucocorticoid sensitivity via direct interaction with GR, at least in a certain gene set that includes *atp1a1* and *scnn1a*.

RESULTS

Dexamethasone (DEX)-Resistance of *atp1a1* and *scnn1a* Not in 293 Cells but in HepG2 Cells

Expression of *atp1a1* and *scnn1a* is shown to be up-regulated by glucocorticoids, as well as aldosterone, in kidney and kidney-derived 293 cells. On the other hand, in liver, their mRNA expression is induced by various intra- or extracellular stimuli except for glucocorticoids (see "Introduction"). That is, *atp1a1* and *scnn1a* appear to be resistant rather selectively against glucocorticoid-GR system particularly in liver. To address the molecular mechanism of such tissue-dependent hormone resistance in gene regulation, we studied HepG2 cells as a model in comparison with 293 cells. When we analyzed their mRNA expression levels using quantitative real-time RT-PCR (qRT-PCR), 6 h treatment of 293 cells with 100 nM DEX induced *atp1a1* and *scnn1a* mRNA expression by 1.9-fold and