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$P2Y_6$ receptor-$G\alpha_{12/13}$ signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis

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Cardiac fibrosis, characterized by excessive deposition of extracellular matrix proteins, is one of the causes of heart failure, and it contributes to the impairment of cardiac function. Fibrosis of various tissues, including the heart, is believed to be regulated by the signalling pathway of angiotensin II (Ang II) and transforming growth factor (TGF)-β. Transgenic expression of inhibitory polypeptides of the heterotrimeric G12 family G protein ($G\alpha_{12/13}$) in cardiomyocytes suppressed pressure overload-induced fibrosis without affecting hypertrophy. The expression of fibrogenic genes (TGF-β, connective tissue growth factor, and periostin) and Ang-converting enzyme (ACE) was suppressed by the functional inhibition of $G\alpha_{12/13}$. The expression of these fibrogenic genes through $G\alpha_{12/13}$ by mechanical stretch was initiated by ATP and UDP released from cardiac myocytes through pannexin hemichannels. Inhibition of G-protein-coupled P2Y6 receptors suppressed the expression of ACE, fibrogenic genes, and cardiac fibrosis. These results indicate that activation of $G\alpha_{12/13}$ in cardiomyocytes by the extracellular nucleotides-stimulated $P2Y_6$ receptor triggers fibrosis in pressure overload-induced cardiac fibrosis, which works as an upstream mediator of the signalling pathway between Ang II and TGF-β.

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Introduction

Heart failure is the final cardiac stage that is observed in nearly all forms of cardiovascular diseases. Structural remodelling of the heart, including myocardial hypertrophy and fibrosis, is a key determinant for the clinical outcome of heart failure (Cohn *et al*, 2000; Berk *et al*, 2007). A variety of evidence indicates that the initial phase in the development of myocardial hypertrophy involves neurohumoral factors, such as endothelin (ET)-1, angiotensin (Ang) II and norepinephrine, and their receptors being coupled to G proteins of the G_q, G_{12}, and G_i families (Sadoshima and Izumo, 1997; Gohla *et al*, 2000; Arai *et al*, 2003). These agonists induce hypertrophic gene expression in cardiomyocytes through the Ca^{2+}-dependent pathway (Onohara *et al*, 2006). Previous studies using transgenic or conditional knockout mice clearly revealed that the G_q family protein predominantly regulates the pathogenesis of hypertrophy (Adams *et al*, 1998; Wettschureck *et al*, 2001). Therefore, it is well recognized that the $G\alpha_q$-mediated Ca^{2+} signalling pathway has an important function in the development of cardiac hypertrophy.

Cardiac fibrosis is characterized by excessive deposition of extracellular matrix (ECM) proteins, such as collagen type I and type III (Brown *et al*, 2005). A variety of growth factors, such as Ang II, ET-1, transforming growth factor (TGF)-β, connective tissue growth factor (CTGF) and periostin, have been reported to promote fibrotic responses of the heart (Katsuragi *et al*, 2004; Zhang *et al*, 2006; Berk *et al*, 2007). Although cardiac fibrosis is accompanied by maladaptive cardiac hypertrophy that eventually results in heart failure, the mechanism of the induction of cardiac fibrosis and its pathophysiological function have yet to be understood.

The relationship between Ang II and TGF-β for induction of fibrosis is a well-established one (Rosenkranz, 2004; Berk *et al*, 2007). In many types of cells, it has been reported that Ang II stimulation regulates TGF-β expression and activation. TGF-β mediates some Ang II-induced responses, and the blockade of Ang II-mediated signalling partially suppresses TGF-β-induced fibrosis (Xu *et al*, 2008). Other signalling molecules for mediating fibrosis are Rho and Rho-associated kinase (ROCK), which are known as downstream effectors of Ang II (Nishida *et al*, 2005). It has been reported that targeted deletion of ROCK suppressed the development of cardiac fibrosis induced by pathological hypertension (Rikitake *et al*, 2005; Zhang *et al*, 2006). ROCK is a downstream mediator of Rho, a small GTP-binding protein (Amano *et al*, 2000), and Rho is reported to exert an effect as one of the downstream mediators of $G\alpha_{12/13}$ (Kozasa *et al*, 1998). We have reported previously that α subunits of the G_{12} family protein ($G\alpha_{12}$ and $G\alpha_{13}$: $G\alpha_{12/13}$) participate in Ang II-, ET-1-, and α_1-adrenergic receptor agonist-induced cardiomyocyte hypertrophy (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). However, the pathophysiological function of $G\alpha_{12/13}$ in cardiac hypertrophy and fibrosis *in vivo* is still unknown.

$G\alpha_{12}$ and $G\alpha_{13}$ appear to be expressed ubiquitously (Simon *et al*, 1991), and a lack of $G\alpha_{13}$ in mice results in embryonic lethality because of the defective organization of the vascular system (Offermanns *et al*, 1997). Therefore, we generated mice with a cardiomyocyte-specific overexpression of $G\alpha_{12/13}$-specific inhibitory polypeptide, which mimics the tissue-

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specific knockout of $G\alpha_{12/13}$. Using these mice, we tested the hypothesis that $G\alpha_{12/13}$ signalling contributes to pressure overload-induced cardiac hypertrophy and associated events *in vivo*.

Results

Cardiac-specific expression of p115-RGS attenuates pressure overload-induced cardiac fibrosis

Previous studies using transgenic or conditional knockout mice clearly revealed that $G\alpha_q$ predominantly regulates the development of hypertrophy (Adams *et al*, 1998; Wettschureck *et al*, 2001). In addition, we reported previously that $G\alpha_{12/13}$ also have an important function in agonist-induced hypertrophic responses of cardiomyocytes using a $G\alpha_{12/13}$-specific inhibitor, a regulator of the G protein signalling domain of p115RhoGEF (p115-RGS) (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). We generated mice with a cardiomyocyte-specific overexpression of p115-RGS protein to test the hypothesis that $G\alpha_{12/13}$ signalling contributes to pressure overload-induced cardiac hypertrophy *in vivo* (Supplementary Figure 1A–C). Ang II stimulation caused Rho activation in the hearts of wild-type (WT) mice, and the activation was completely suppressed in transgenic (p115-Tg) mice (Supplementary Figure 1D). This result confirmed that receptor-stimulated activation of $G\alpha_{12/13}$ signalling is inhibited in the p115-Tg heart. Pressure overload was induced by surgical transverse aortic constriction (TAC)

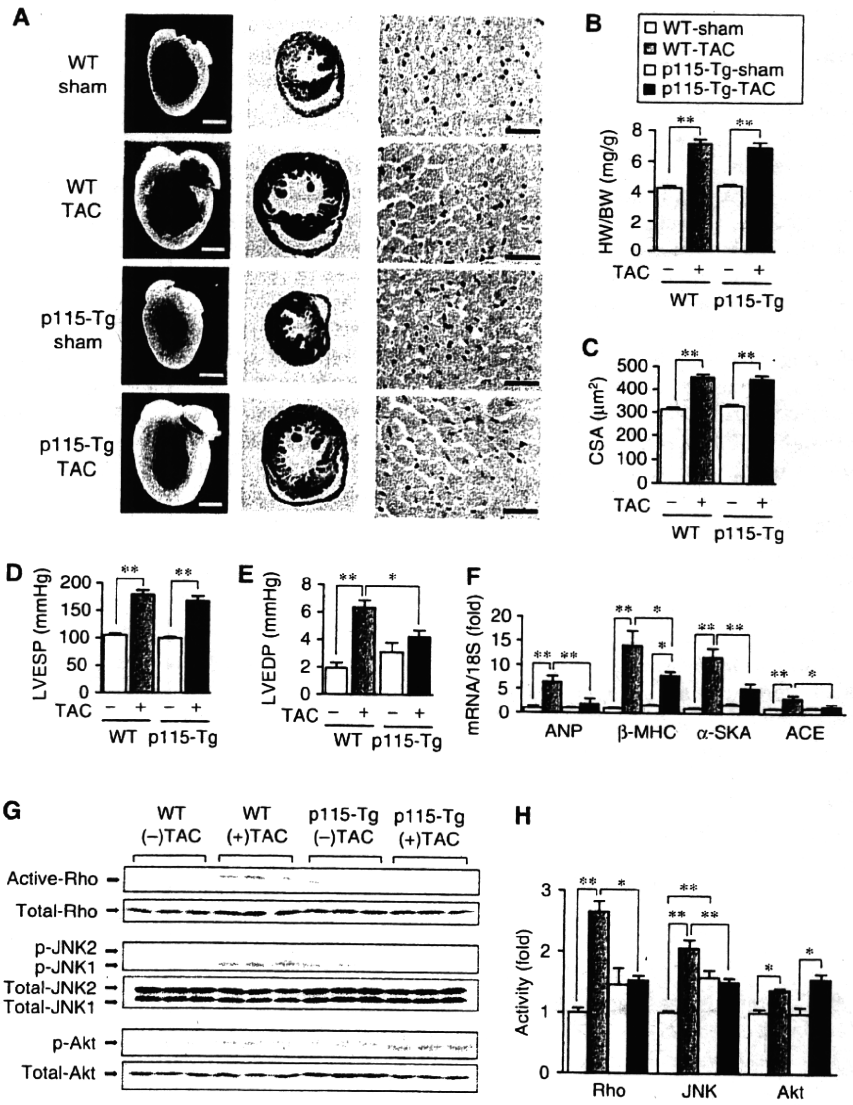

Figure 1 p115-RGS suppresses pressure overload-induced cardiac dysfunction but not hypertrophy. (**A–F**) TAC-induced increase in the size of the heart and cardiomyocytes (A–C), left ventricular (LV) pressure (D, E), and the expression of hypertrophic genes (F) in WT and p115-Tg mice. (A) Gross appearance of the hearts and H&E-stained mid-transverse sections of hearts isolated from WT and p115-Tg mice 6 weeks after sham or TAC surgery. Left (white) scale bar, 2 mm; right (black) scale bar, 30 μm. (B and C) Heart weight (HW) to body weight (BW) ratios (B) and cross-sectional areas (CSA) of cardiomyocytes (C). (D and E) LV pressure. LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure. (F) Expression of ANP, β-MHC, α-SKA, and ACE mRNAs. (**G, H**) Activation of Rho, JNK, and Akt induced by 1 week of TAC in WT and p115-Tg hearts. Error bars indicate s.e.m.; $n=3$–5 (C), $n=17$–23 (B, D, E), $n=3$ (F, H). Representative result of hearts from sham ($n=3$) and TAC surgery ($n=5$) was shown (A). * indicates $P<0.05$ and ** indicates $P<0.01$.

in WT and p115-Tg mice. The increase in size of the p115-Tg heart is essentially the same as that in WT mice (Figure 1A–C). TAC of p115-Tg mice increased left ventricular end-systolic pressure (LVESP) to the same extent as that in WT mice (Figure 1D), indicating that pressure overload by TAC was equally performed. TAC induced a significant elevation of left ventricular end-diastolic pressure (LVEDP) in WT mice. However, there was no alteration in p115-Tg mice (Figure 1E). Although the LV systolic function in p115-Tg mice was slightly reduced in sham operation compared with that in WT mice, there was no further impairment by TAC (Figure 1E and Supplementary Table 1). These results suggest that systolic and diastolic function of the p115-Tg heart is not impaired after TAC. TAC in WT mice strongly increased the expression of messenger ribonucleic acid (mRNA) of classical markers of pathological hypertrophy in myocardium, atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and α-skeletal muscle actin (α-SKA) (Figure 1F). However, the expression of these genes in p115-Tg hearts was less than half of that in WT hearts. We have reported that $G\alpha_{12/13}$ mediate activation of Rho and c-Jun NH_2-terminal kinase (JNK) in cultured cardiomyocytes (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). Pressure overload increased the activities of Rho and JNK in WT hearts, but the activation of Rho and JNK was significantly suppressed in TAC of p115-Tg hearts (Figure 1G and H). However, TAC-induced Akt activation was not suppressed in p115-Tg hearts, suggesting that p115-RGS specifically inhibits $G\alpha_{12/13}$-mediated signalling in the heart. As the TAC-induced Rac activation was also suppressed in p115-Tg hearts (Supplementary Figure 1E), $G\alpha_{12/13}$ may mediate TAC-induced JNK activation through Rho- and Rac-dependent pathways in mouse hearts as well as rat cardiac myocytes.

As overproduction of ECM protein causes ventricular stiffness leading to the impairment of diastolic function (Berk *et al*, 2007), we examined the involvement of $G\alpha_{12/13}$ in pressure overload-induced cardiac fibrosis. TAC increased the expression of collagen types I and III proteins in the interstitial tissue in WT mice, as determined by picrosirius red staining (Figure 2A). In contrast, TAC-induced collagen deposition in p115-Tg mice was less than half of that in WTs (Figure 2B). The relationship between heart weight to body weight ratio (HW/BW) and collagen expression in WT hearts reveals that the degree of hypertrophy correlates positively with severity of fibrosis (Figure 2C). In contrast, the correlation between HW/BW and collagen expression in p115-Tg hearts also indicates that TAC-induced cardiac fibrosis was significantly suppressed despite the development of cardiac hypertrophy. TAC increased the expression of mRNAs for procollagen type I and type III, fibrogenic factors (CTGF (Hahn *et al*, 2000), periostin (Katsuragi *et al*, 2004), and TGF-βs (Zhang *et al*, 2000)) in WT hearts (Figure 2D). These increases were significantly attenuated in p115-Tg hearts. Furthermore, TAC increased the expression of periostin, mature TGF-βs, and angiotensin-converting enzyme (ACE) in WT hearts, but these increases were attenuated also in p115-Tg hearts (Figure 2E). It has been

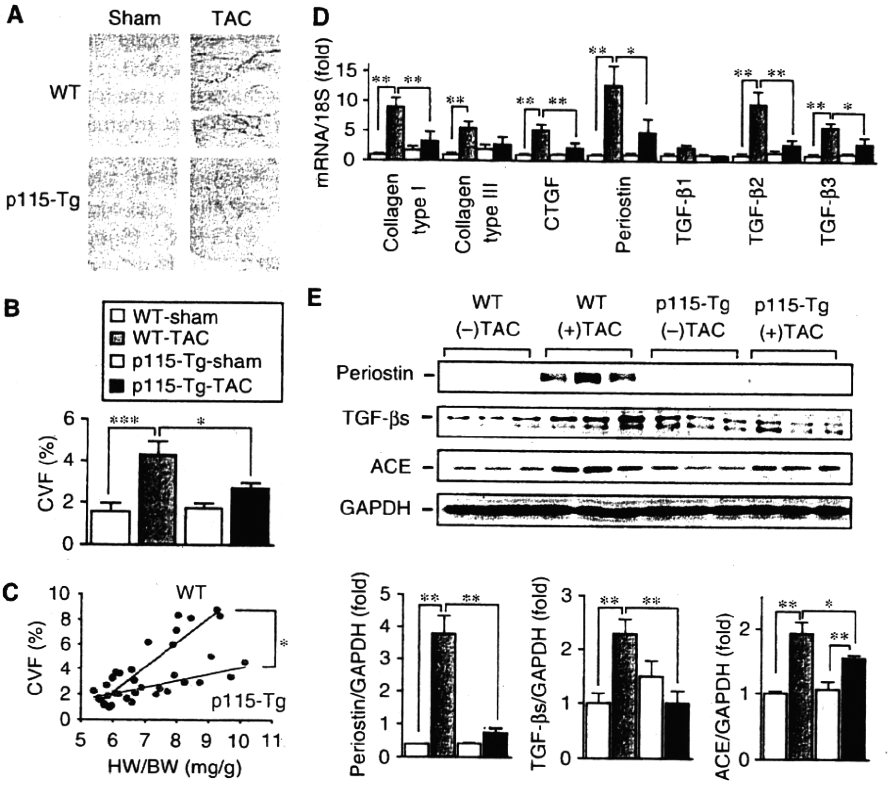

Figure 2 $G\alpha_{12/13}$ mediate pressure overload-induced cardiac fibrosis. (**A**) LV sections stained by picrosirius red. (**B**, **C**) Collagen volume fraction (CVF) (B) and correlations between HW/BW and CVF (C). (**D**) Expression of collagen type I, type III, CTGF, periostin, and TGF-β1-3 mRNAs. (**E**) Expression of periostin, mature TGF-βs, and ACE proteins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels were used as an internal control. Error bars indicate s.e.m.; $n=15$–19 (B), $n=3$ (D, E). Representative picrosirius red staining sections of hearts from WT ($n=15$–19) and p115-Tg ($n=15$–16) mice were shown (A). * indicates $P<0.05$ and ** indicates $P<0.01$.

postulated that activation of the renin–angiotensin–aldosterone system (RAAS) and increased levels of active TGF-β1 has an important function in pressure overload-induced cardiac fibrosis (Berk *et al*, 2007). Pressure overload increased three TGF-β mRNA isoforms and proteins in WT hearts, and these increases were suppressed in p115-Tg hearts. These results suggest that $G\alpha_{12/13}$ mediate pressure overload-induced cardiac fibrosis by an increase in induction of CTGF, periostin, and TGF-βs.

Activation of $G\alpha_{13}$ in cardiomyocytes induces cardiac fibrosis

We also generated mice with cardiomyocyte-specific overexpression of a constitutively active (CA) mutant of $G\alpha_{13}$ protein (CA-$G\alpha_{13}$) (Supplementary Figure 2). The CA-$G\alpha_{13}$ heterozygous mice did not show an increase in heart size, compared with WT mice (Supplementary Figure 3A and B). However, collagen deposition was significantly increased (Supplementary Figure 3C). The expression of mRNA for CTGF was strongly increased in CA-$G\alpha_{13}$ mice (Supplementary Figure 3D). The expression of mRNA for ACE and protein expression were slightly increased (Supplementary Figure 3E). The expression of mRNAs for periostin and TGF-βs was not increased, but proteins of periostin and TGF-βs were increased in CA-$G\alpha_{13}$ hearts. As CA-$G\alpha_{13}$ increased proteins but not mRNAs for periostin and TGF-βs, $G\alpha_{13}$ may participate in the stabilizing of periostin and TGF-βs proteins. These results also suggest that pressure overload-induced expression of fibrogenic factors is mediated by $G\alpha_{13}$. In contrast to CTGF, the expression of hypertrophy-related genes (ANP and β-MHC) was not increased in CA-$G\alpha_{13}$ heart, consistent with the inability of CA-$G\alpha_{13}$ to induce hypertrophy. However, strong activation of $G\alpha_{13}$ signalling may induce cardiac hypertrophy as well as fibrosis, as CA-$G\alpha_{13}$ homozygous mice showed a significant increase in heart size (data not shown). The LV function of CA-$G\alpha_{13}$ mice was significantly decreased compared with that of WT mice (Supplementary Figure 3F and G). These results suggest that $G\alpha_{12/13}$ mediate cardiac fibrosis and dysfunction induced by pressure overload.

Extracellular nucleotides mediate mechanical stretch-induced $G\alpha_{12/13}$ activation through purinergic receptors

As heterotrimeric G proteins are activated primarily by receptor stimulation, it is reasonable to assume that pressure overload activates $G\alpha_{12/13}$-coupled receptors. As mechanical stretch of cardiomyocytes is frequently used as an *in vitro* model of pressure overload, we examined which G protein-coupled receptor(s) are involved in mechanical stress-induced $G\alpha_{12/13}$ activation. As activation of small GTP-binding protein Rho is a sensitive marker of $G\alpha_{12/13}$ activity (Kozasa *et al*, 1998), we measured Rho activity as an index of the magnitude of $G\alpha_{12/13}$ signalling. Mechanical stretch of cardiomyocytes increased Rho activity, and this increase was sustained for 30 min (Figure 3A). Overexpression of p115-RGS completely inhibited mechanical stretch-induced Rho activation at early time and 30 min (Figure 3B and C). As a mutation in the RGS domain of p115RhoGEF loses the interaction with $G\alpha_{12/13}$ (Bhattacharyya and Wedegaertner, 2003), we expressed the mutated p115-RGS to examine whether the effects of p115-RGS are specific for inhibition of interaction with $G\alpha_{12/13}$. Expression of the interaction-deficient mutant of p115-RGS did not affect mechanical stretch-induced Rho activation. In addition, treatment with *Pertussis* toxin, an uncoupler of receptor-G_i interaction, did not suppress mechanical stretch-induced Rho activation. These results suggest that mechanical stretch activates Rho through $G\alpha_{12/13}$. It has been reported that Ang type 1 receptor (AT1R) is activated by mechanical stretch without the involvement of Ang II, and AT1R antagonist blocks mechanical stretch-induced G_q activation and hypertrophic responses (Zou *et al*, 2004). However, mechanical stretch-induced Rho activation through $G\alpha_{12/13}$ was not attenuated by treatment with not only CV11974 (AT1R antagonist) but also PD123319 (AT2R antagonist), propranolol (β adrenergic receptor (AR) antagonist), prazosin (α_1AR antagonist), BQ123 (ET type A receptor antagonist), BQ788 (ET type B receptor antagonist) and CGP20712A (selective β_1AR antagonist) (Figure 3C and Supplementary Figure 4A and B). Mechanical stretch increases intracellular Ca^{2+} concentration through mechanosensitive cation channels (Christensen and Corey, 2007). However, treatment with an inhibitor of stretch-sensitive channels GsMTx4, intracellular Ca^{2+} chelator BAPTA-AM, and L-type Ca^{2+} channel blocker nitrendipine did not suppress mechanical stretch-induced Rho activation (Figure 3C and D). The Src family kinase substrate p130Cas has been reported to function as a mechanosensor (Sawada *et al*, 2006), but an Src inhibitor, PP2, did not affect mechanical stretch-induced Rho activation (data not shown). In contrast, treatment with apyrase, an ATP/ADP scavenging enzyme, completely blocked mechanical stretch-induced Rho activation (Figure 3D and Supplementary Figure 4C). Treatment with another ATP scavenging enzyme, hexokinase II, or purinergic receptor antagonists, suramin and PPADS, also suppressed mechanical stretch-induced Rho activation. Furthermore, extracellular treatment with ATP, ADP, UTP, and UDP, but not adenosine, increased Rho activity (Supplementary Figure 4D–F). The extracellular nucleotide-stimulated Rho activation was completely suppressed by the expression of p115-RGS (Supplementary Figure 4G). Mechanical stretch actually activated $G\alpha_{12}$ and $G\alpha_{13}$, which were completely suppressed by treatment with suramin (Figure 3E and F). These results suggest that extracellular nucleotides mediate mechanical stretch-induced $G\alpha_{12/13}$ activation through purinergic receptors in rat cardiomyocytes.

Pannexin-1 mediates mechanical stretch-induced release of nucleotides

Extracellular ATP in the cardiovascular system may originate from different cellular sources, such as perivascular sympathetic nerve endings (Burnstock, 1972), activated platelets, endothelial cells, and inflammatory cells. It has also been postulated that connexin and pannexin hemichannels are involved in ATP release caused by mechanical stimulation in cardiac myocytes (Suadicani *et al*, 2000; Shestopalov and Panchin, 2008). Mechanical stretch of cardiomyocytes increased extracellular ATP concentration (Figure 4A). Treatment with hemichannel inhibitors, carbenoxolone, and 1-heptanol, suppressed both mechanical stretch-induced Rho activation and the increase in extracellular ATP concentration (Figures 3D and 4B). As the increase in extracellular ATP was not affected by p115-RGS and P2 receptor antagonists (PPADS and suramin), $G\alpha_{12/13}$ do not participate in ATP release, but rather mediate mechanical stretch-induced Rho activation.

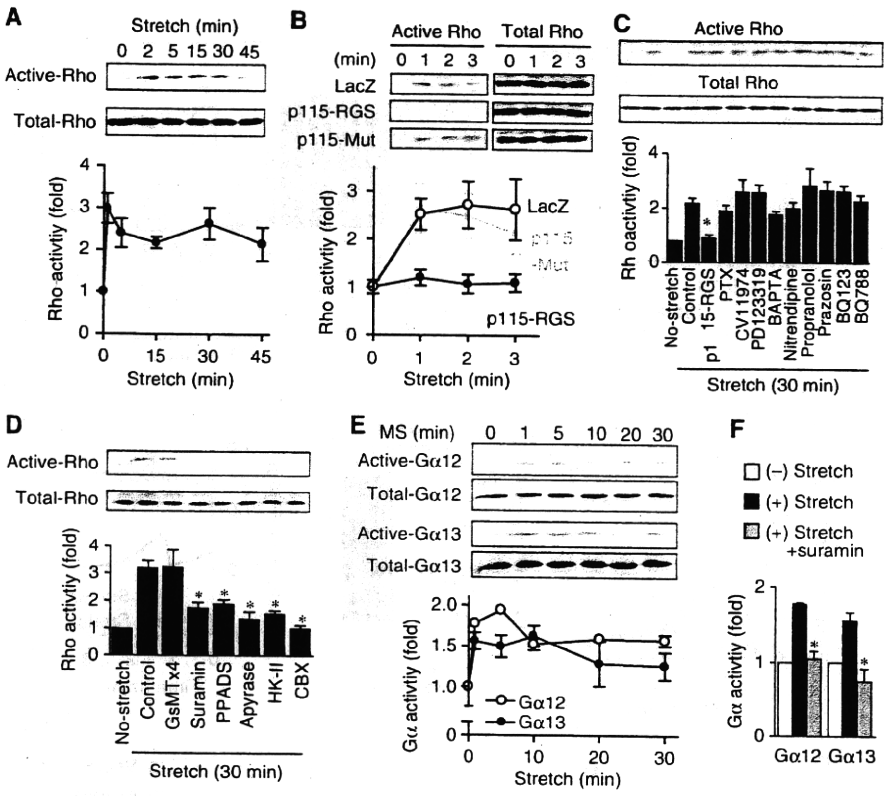

Figure 3 Mechanical stretch activates $G\alpha_{12/13}$ and Rho through purinergic receptor. (**A**) Time course of Rho activation by mechanical stretch. (**B**) $G\alpha_{12/13}$-mediated Rho activation by mechanical stretch. Cells were transfected with GFP, p115-RGS, and inactive mutant of p115-RGS (p115-Mut) by electroporation. (**C, D**) Effects of various inhibitors on Rho activation by mechanical stretch. Cells were treated with CV11974 (2.5 μM), PD123319 (2.5 μM), BAPTA-AM (3 μM), nitrendipine (1 μM), propranolol (1 μM), prazosin (10 μM), BQ123 (3 μM), BQ788 (3 μM), GsMTx4 (1 μM), apyrase (1 U/ml), hexokinase (HK)-II (100 μg/ml), suramin (100 μM), PPADS (100 μM), carbenoxolone (CBX; 20 μM), and *Pertussis* toxin (PTX; 100 ng/ml for 12 h) 5 min before mechanical stretch. (**E**) Time courses of $G\alpha_{12}$ and $G\alpha_{13}$ activation by mechanical stretch (MS). (**F**) Effects of suramin on $G\alpha_{12}$ and $G\alpha_{13}$ activation. Cells were pretreated with suramin (100 μM) 5 min before mechanical stretch. Error bars indicate s.e.m.; $n=4$ (A, D) and $n=3$ (B, C, E, F). * indicates $P<0.05$ versus control.

The function of all connexins as gap junction channels or hemichannels is strongly dependent on Ca^{2+}, but the function of pannexin-1 is independent of Ca^{2+} (Shestopalov and Panchin, 2008). As mechanical stretch-induced Rho activation was independent of Ca^{2+} (Figure 3C) and a low concentration of carbenoxolone (but not 1-heptanol) inhibited mechanical stretch-induced ATP release (Figure 4B), pannexin-1 appears to be a prime candidate for an ATP release channel. Pannexin-1 and pannexin-2 mRNAs, but not pannexin-3 mRNA, were expressed in mouse hearts and rat cardiomyocytes (Supplementary Figure 5). The expression of pannexin-1 mRNA was increased by pressure overload (Supplementary Figure 5A). Treatment with siRNAs for pannexin-1 induced a 50% decrease in pannexin-1 mRNA levels (Supplementary Figure 5B). The mechanical stretch-induced ATP release was decreased by about 50% in pannexin-1 siRNA-treated cardiomyocytes (Figure 4C). These results suggest that pannexin-1 mediates ATP release by mechanical stretch in rat cardiomyocytes.

Involvement of $P2Y_6$ receptor in mechanical stretch-induced fibrotic responses

We also examined which receptor subtype(s) is involved in mechanical stretch-induced $G\alpha_{12/13}$ activation. RT–PCR analysis showed that mouse hearts express mRNAs coding $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ receptors (Supplementary Figure 6). Among them, mRNA levels of $P2Y_2$ and $P2Y_6$ receptors were upregulated in TAC hearts and CA-$G\alpha_{13}$ hearts. We also found that neonatal cardiomyocytes express mRNAs coding $P2Y_1$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors (data not shown). Treatment with MRS2578, a selective $P2Y_6$ receptor antagonist, suppressed mechanical stretch-induced Rho activation in a concentration-dependent manner, with an IC_{50} value of about 0.1 μM (Figure 5A and B). In contrast, treatment with MRS2179 (a $P2Y_1$ receptor antagonist), AR-C67719MX (a $P2Y_{12}$ receptor antagonist), and 8-SPT (an adenosine receptor antagonist) did not suppress mechanical stretch-induced Rho activation. As Rho is reported to regulate the expression levels of CTGF (Hahn *et al*, 2000) and periostin (Butcher *et al*, 2007), we examined the effects of P2Y receptor antagonists on the expression of these fibrogenic factors. Mechanical stretch increased expression of CTGF mRNA, which had been completely suppressed by the expression of p115-RGS, and by treatment with suramin, PPADS, and MRS2578 (Figure 5C and D). Mechanical stretch increased the expression of TGF-β2 mRNA but did not affect the expression of TGF-β1 and -β3 mRNAs, and the induction of TGF-β2 mRNA was also suppressed by suramin, PPADS, and MRS2578. In addition, mechanical stretch increased periostin proteins two-fold, which had been completely suppressed

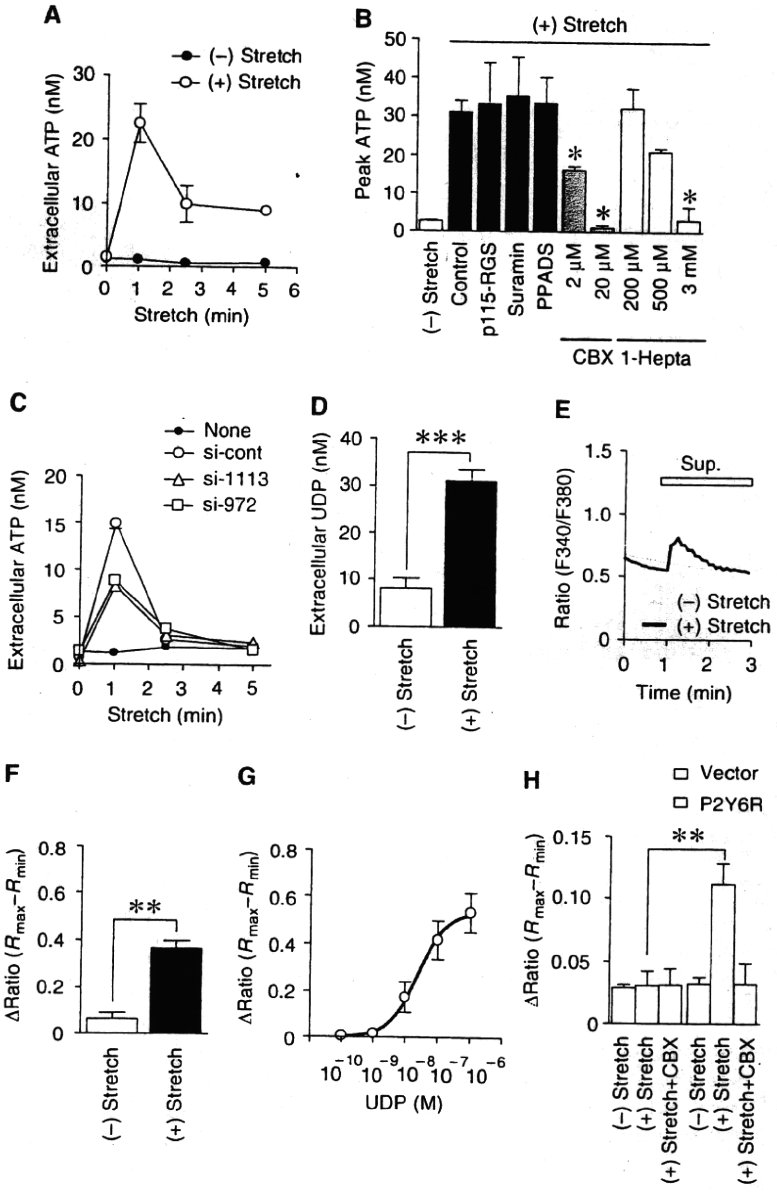

Figure 4 Pannexin 1 mediates mechanical stretch-induced release of nucleotides. (**A**) Time course of ATP release by mechanical stretch. Cells were treated with ARL67156 (50 μM) 20 min before mechanical stretch. (**B**) Effects of inhibitors on the increase in extracellular ATP concentration induced by mechanical stretch. Cells were treated with suramin (100 μM), PPADS (100 μM), carbenoxolone (CBX), and 1-heptanol (1-Hepta) 20 min before mechanical stretch. (**C**) Effects of pannexin-1 siRNAs on mechanical stretch-induced ATP release. (**D**) UDP concentration in the culture medium from control cardiomyocytes ((−) stretch) or mechanically stretched cells for 10 min ((+) stretch). (**E, F**) Time courses of changes (E) and peak increases (F) in $[Ca^{2+}]_i$ of $P2Y_6$ receptor-expressing HEK293 cells. The changes in $[Ca^{2+}]_i$ were determined after the substitution of external solution with the culture medium (Sup.) of mechanically stretched cells. (**G**) Concentration-dependent maximal increases in $[Ca^{2+}]_i$ induced by UDP in $P2Y_6$ receptor-expressing HEK293 cells. (**H**) Peak increases in $[Ca^{2+}]_i$ of control (vector) and $P2Y_6$ receptor-expressing H9c2 myoblasts. The changes in $[Ca^{2+}]_i$ were determined after the addition of the culture medium from control cardiomyocytes ((−) stretch) or mechanically stretched cells ((+) stretch) for 10 min with or without 2 μM of carbenoxolone (+CBX). Error bars indicate s.e.m.; $n=4$ (A) and $n=3$ (B–H). * indicates $P<0.05$, ** indicates $P<0.01$ and *** indicates $P<0.001$.

by the expression of p115-RGS, and by treatment with suramin, PPADS, and MRS2578 (Figure 5E). These increases were not affected by the expression of G protein-coupled receptor kinase 2-RGS, a $G\alpha_q$-specific RGS domain (Nishida *et al*, 2005; Onohara *et al*, 2006), nor by the treatment with $P2Y_1$ receptor antagonist (MRS2179), $P2Y_{12}$ receptor antagonist (AR-C67719MX), and 8-SPT (an adenosine receptor antagonist). As $P2Y_2$ receptor-selective antagonist is not commercially available, we examined the involvement of the $P2Y_2$ receptor in mechanical stretch-induced Rho activation with siRNAs. The treatment with $P2Y_2$-specific siRNAs decreased the mRNA by about 50% but did not suppress Rho activity (Figure 5F). In contrast, the treatment with $P2Y_6$-specific siRNAs decreased the mRNA by about 70% and significantly suppressed mechanical stretch-induced Rho activation in cardiomyocytes. These results suggest

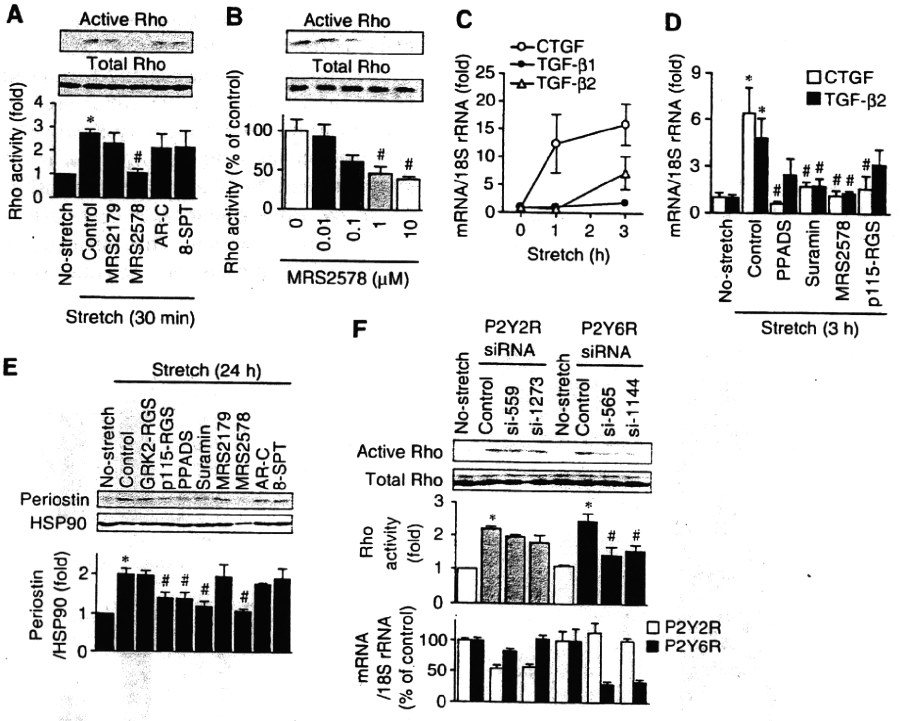

Figure 5 Involvement of $P2Y_6$ receptor in mechanical stretch-induced Rho activation and expression of fibrogenic factors. (**A**) Effects of MRS2179 ($P2Y_1$ receptor antagonist, 10 μM), MRS2578 ($P2Y_6$ receptor antagonist, 10 μM), AR-C69931MX ($P2Y_{12}$ receptor antagonist, AR-C; 1 μM), and 8-(p-sulphophenyl) theophylline (8-SPT; adenosine receptor antagonist, 10 μM) on Rho activation. (**B**) Concentration-dependent inhibition of Rho activation by MRS2578. (**C**) Time courses of changes in CTGF and TGF-β1 and -β2 mRNA expressions induced by mechanical stretch. (**D**) Effects of inhibitors on the expression of CTGF and TGF-β2 mRNAs induced by mechanical stretch for 3 h. (**E**) Effects of various inhibitors on the expression of periostin induced by mechanical stretch for 24 h. The expression was plotted by the ratio to HSP90. (**F**) Effects of siRNAs of $P2Y_2$ and $P2Y_6$ receptors on mechanical stretch-induced Rho activation. Lower graph shows the expression levels of $P2Y_2$ and $P2Y_6$ receptor mRNAs. Error bars indicate s.e.m.; $n=3$ (A, B, D, E), $n=5$ (C) and $n=6$ (F). * indicates $P<0.05$ versus no-stretch and # indicates $P<0.05$ versus control.

that the $P2Y_6$ receptor predominantly regulates the mechanical stretch-induced activation of fibrotic signalling in cardiomyocytes.

As the $P2Y_6$ receptor is mainly activated by UDP (Vassort, 2001), and uridine nucleotides are known to be released by mechanical stretch (Lazarowski and Boucher, 2001), we also examined whether UDP is released by mechanical stretch. Mechanical stretch of cardiomyocytes increased extracellular UDP concentration three-fold (Figure 4D). In addition, treatment of $P2Y_6$ receptor-overexpressing HEK293 cells with supernatant from mechanically stretched rat cardiomyocytes significantly increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Figure 4E). The magnitude of maximal increase in $[Ca^{2+}]_i$ induced by the supernatant was equivalent to the peak $[Ca^{2+}]_i$ increase induced by 30 nM of extracellular UDP (Figure 4F and G). As H9c2 myofibroblasts do not express $P2Y_1$ and $P2Y_2$ receptors, we further examined the effects of nucleotides on $[Ca^{2+}]_i$ increase using H9c2 cells. Treatment of vector-expressing H9c2 cells with UDP, ATP, or the supernatant of stretch-activated cardiomyocytes did not show any significant increases in $[Ca^{2+}]_i$, but the treatment with the supernatant significantly increased $[Ca^{2+}]_i$ in $P2Y_6$ receptor-overexpressing H9c2 cells (Figure 4H). This $[Ca^{2+}]_i$ increase was completely suppressed by the treatment of cardiomyocytes with carbenoxolone, suggesting that pannexin-1 mediates mechanical stretch-induced UDP release. Furthermore, treatment of cardiomyocytes with 3-phenacyl-UDP, a highly selective $P2Y_6$ receptor agonist, increased Rho activity in a concentration-dependent manner (Supplementary Figure 4H). These results suggest that extracellular UDP predominantly mediates mechanical stretch-induced $P2Y_6$ receptor activation in cardiomyocytes.

Inhibition of $P2Y_6$ receptors attenuates pressure overload-induced cardiac fibrosis in vivo

We next examined whether purinergic receptors actually participate in pressure overload-induced cardiac fibrosis *in vivo*. Treatment with MRS2578 after TAC significantly suppressed pressure overload-induced collagen deposition without affecting cardiomyocyte hypertrophy (Figure 6A–C). Treatment with MRS2578 significantly suppressed LV dysfunction induced by pressure overload (Figure 6D and E and Supplementary Table 3). Furthermore, the treatment with MRS2578 suppressed the increases in mRNA expressions of ANP, β-MHC, procollagen type I, periostin, and TGF-β2 by pressure overload (Figure 6F). We also found that MRS2578 inhibited pressure overload-induced Rho activation and TAC-induced increases in expression of periostin, mature TGF-βs, and ACE proteins (Figure 6G and H). Furthermore, we found that treatment with suramin also suppressed pressure overload-induced collagen deposition and LV dysfunction (Supplementary Figure 7 and Supplementary Table 4). These results suggest that inhibition of $P2Y_6$ receptors

actually attenuates pressure overload-induced cardiac fibrosis and LV dysfunction.

Discussion

Remodelling of the heart, including accumulation of ECM and an associated change in ventricular geometry, is a common feature of heart failure. In this study, we found that $G\alpha_{12/13}$ mediate cardiac fibrosis without the development of hypertrophy induced by pressure overload. We reported previously that $G\alpha_{12/13}$ mediate agonist-induced hypertrophic responses of cardiomyocytes. However, we also found that mechanical stretch-induced increases in NFAT- and BNP-dependent transcriptional activities are not suppressed in p115-RGS-expressing myocytes (Supplementary Figure 8). These results suggest that activation of $G\alpha_{12/13}$ is not involved in mechanical stress-induced NFAT and BNP expression. $G\alpha_{12/13}$ are activated by extracellular ATP and UDP that are released by mechanical stretch. The nucleotides released through pannexin-1 hemichannels activate $G\alpha_{12/13}$-mediated Rho

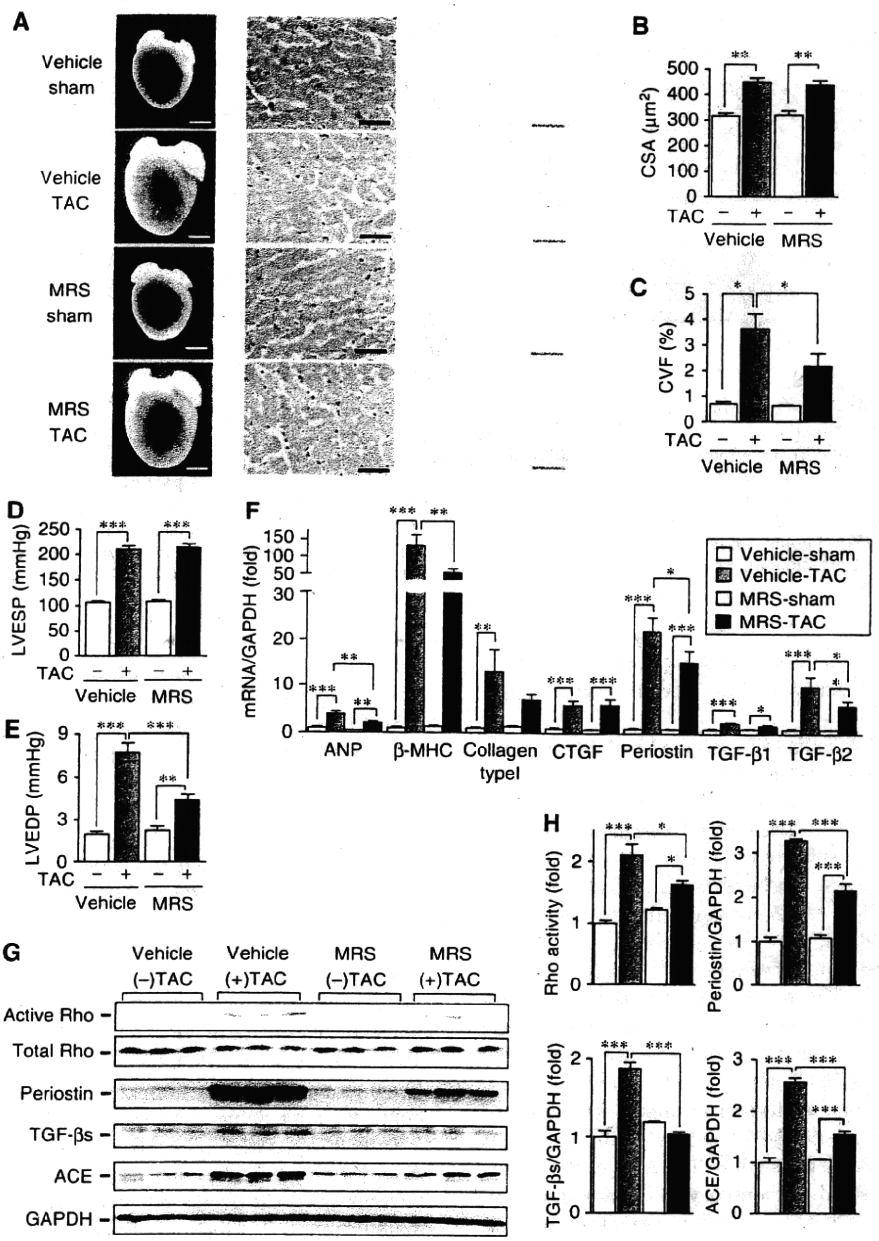

Figure 6 Involvement of $P2Y_6$ receptors in pressure overload-induced cardiac fibrosis. Effects of MRS2578 (3 mg/kg/day, started from 3 days after TAC surgery) on TAC-induced fibrosis and hypertrophy (**A–C**), and LV functions (**D**, **E**). (A) Hearts (left; scale bar, 2 mm), H&E staining (center; scale bar, 30 μm), and picrosirius red staining (right; scale bar, 200 μm). (B) CSA of cardiomyocytes. (C) Results of cardiac fibrosis. (**F**) Expression of hypertrophic and fibrogenic genes. (**G**) Effects of MRS2678 on TAC-induced Rho activation and the expression of periostin, mature TGF-βs, ACE, and GAPDH. GAPDH was used as an internal control. Error bars indicate s.e.m.; $n=3$–8 (B, C), $n=8$–12 (D, E), $n=5$–7 (F), and $n=3$ (G, **H**). Representative result of hearts from vehicle-sham ($n=3$), vehicle-TAC ($n=7$), and MRS-sham ($n=3$), and MRS-TAC mice ($n=8$) was shown (A). * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.

activation leading to the induction of fibrogenic factors, such as CTGF and periostin. Furthermore, inhibition of purinergic receptors attenuates the TAC-induced cardiac fibrosis and LV dysfunction. These results indicate that activation of $G_{12/13}$-coupled purinergic receptors in cardiomyocytes by extracellular nucleotides stimulate the secretion of fibrogenic factors and trigger pressure overload-induced cardiac fibrosis (Figure 7). Purinergic receptors are classified into two families: P2X and P2Y. P2X receptors are transmitter-gated channels and consist of 7 subtypes. P2Y receptors are G protein-coupled receptors and are divided into eight subtypes. We found that the $P2Y_6$ receptor predominantly regulates mechanical stretch-induced Rho activation and the expression of fibrogenic factors in rat cardiac myocytes (Figure 5). We also found that inhibition of $P2Y_6$ receptors suppressed cardiac fibrosis and diastolic dysfunction induced by pressure overload (Figure 6). These results suggest that $P2Y_6$ receptors in cardiomyocytes have an important function in pressure overload-induced cardiac fibrosis.

It has been reported that CTGF has an important function in cardiac fibrosis. In contrast to CTGF, the function of periostin remains to be determined. Extracellular application of periostin induced re-entry of cardiomyocytes into the cell cycle, and reduced fibrosis whereas improving cardiac functions (Kühn *et al*, 2007). However, analysis of knockout and transgenic mice reveals that periostin is involved in myocardial infarction-induced fibrosis and impairment of ventricular functions (Oka *et al*, 2007; Shimazaki *et al*, 2008). They also demonstrated that pressure overload-induced hypertrophic responses and fibrosis are regulated by periostin. The present results are consistent with the findings that periostin is involved in pressure-overload-induced cardiac fibrosis.

It is interesting to note that the $G_{12/13}$-mediated pathway regulates fibrosis, and the $G_{q/11}$-mediated pathway regulates hypertrophy. Two different G proteins regulate two distinct responses: fibrosis and hypertrophy. Many groups using transgenic and knockout mice have reported that suppression of hypertrophy leads to the inhibition of fibrosis. However, we demonstrated that fibrosis and hypertrophy are independent processes, as revealed by expressing p115-RGS to block $G\alpha_{12/13}$ functions. Therefore, $G\alpha_{12/13}$-mediated signalling leading to cardiac fibrosis may turn on after hypertrophy is already developed. This speculation is supported by the finding that pannexin-1 mRNA in the heart is upregulated by pressure overload (Supplementary Figure 5A). Thus, the process of the hypertrophied heart depositing ECM proteins *in vivo* may be triggered by the release of ATP and UDP from myocytes during transition from hypertrophy to heart failure.

There are three structurally distinct TGF-βs (Bujak and Frangogiannis, 2007). TGF-β1 is a prevalent isoform, and TGF-β2 and -β3 are expressed in limited tissues. As these three isoforms do not compensate for functions of other isoforms, each TGF-β has specific and independent roles *in vivo*. Among these three isoforms, it has been reported that TGF-β1 mediates Ang II-induced hypertrophic responses *in vivo* (Schultz Jel *et al*, 2002). Myocardial infarction increases the expression of these three TGF-β isoforms, which participate in inflammation at an early phase and cardiac remodelling at a later phase. We found that TGF-β2 mRNA was most responsive to TAC, which induces cardiac fibrosis (Figure 1). We also demonstrated that p115-RGS and the $P2Y_6$ receptor antagonist inhibit the expression of TGF-β2 mRNA by pressure overload (Figure 2 and Figure 6). TGF-β2 may be the predominant form of TGF-β for the promotion of fibrosis in the heart.

Our results also indicate that $G\alpha_{13}$ mediates pressure overload-induced expression of ACE proteins (Figure 1 and Supplementary Figure 3). Although inhibition of ACE expression has been reported to inhibit pressure overload-induced cardiac hypertrophy in rats (Baker *et al*, 1990; Zierhut *et al*, 1991), inhibition of $G\alpha_{12/13}$, upstream of ACE, did not suppress cardiac hypertrophy in mice (Figure 1). We do not have any data to explain this discrepancy. However, our data are consistent with the results of Xiao *et al*. (2008), which show that an increase in ACE expression does not augment pressure overload-induced cardiac hypertrophy in mice. In addition, pressure overload induces cardiac hypertrophy in angiotensinogen-knockout mice (Zou *et al*, 2004). Crowley *et al* (2006) have reported that Ang II induces cardiac hypertrophy in mice through stimulation of AT1 receptors in the kidney. It has been reported that the expression of a gain-of-function mutant of Ang II type 1A receptor in the

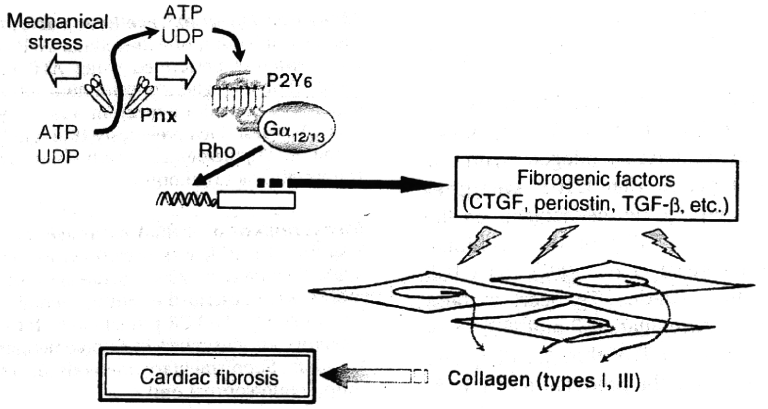

Figure 7 Schema for the mechanism of cardiac fibrosis induced by pressure overload. Mechanical stretch induces release of nucleotides through pannexin-1 hemichannels (Pnx) from cardiac myocytes, which leads to stimulation of purinergic $P2Y_6$ receptors. Nucleotide-bound $P2Y_6$ receptor activates $G_{12/13}$ proteins. Activation of $G\alpha_{12/13}$ in cardiomyocytes induces the expression of fibrogenic factors, which activate cardiac fibroblasts in a paracrine manner. The activated fibroblasts produce excessive amount of collagen types I and III, leading to induction of cardiac fibrosis.

heart causes cardiac fibrosis but not hypertrophy (Billet *et al*, 2007). Thus, increase in cardiac ACE activity induced by pressure overload may not contribute to the development of cardiac hypertrophy in mice. Using rat cardiomyocytes, we found that mechanical stretch-induced activation of JNK and p38 MAPK, but not ERK, was suppressed by p115-RGS (Supplementary Figure 8). As ERK, but not JNK and p38 MAPK, participates in cardiac hypertrophy (Liang and Molkentin, 2003), $G\alpha_{12/13}$-mediated ACE expression may participate in mechanical stress (pressure overload)-induced hypertrophy in rats but not mice.

Cardiac fibrosis is considered one of the inflammatory responses of the heart (Brown *et al*, 2005). A variety of evidence supports the idea that extracellular nucleotides function as a mediator of inflammatory responses, such as chemotaxis and phagocytosis (Chen *et al*, 2006; Idzko *et al*, 2007; Koizumi *et al*, 2007). Our data suggest that extracellular nucleotides function as a priming factor in the development of cardiac fibrosis induced by pressure overload. It is generally thought that activation of the RAAS system and increased levels of active TGF-β stimulate cardiac fibroblasts and induce ECM deposition, leading to perivascular fibrosis. CTGF, periostin, and TGF-β2 mRNAs were upregulated by pressure overload, and the increased expression of three genes were suppressed in p115-Tg mice. Furthermore, a $P2Y_6$ receptor antagonist MRS2578 suppressed the stress-induced expression of periostin and TGF-β mRNAs *in vitro* and *in vivo*. In addition to TGF-β, we demonstrate that CTGF and periostin are also involved in pressure overload-induced cardiac fibrosis. As $G\alpha_{12/13}$ mediate cardiac fibrosis, which is associated with pressure overload-induced hypertrophy, the development of drugs to block $P2Y_6$ receptors-$G\alpha_{12/13}$ signalling may be a novel strategy for heart failure.

The interrelationship between Ang II and TGF-β is well established. The blockade of TGF-β by an antibody and a mutated TGF-receptor suppressed some of Ang II-induced responses (Bujak and Frangogiannis, 2007). Therefore, it is reasonable to assume that Ang II stimulates TGF-β expression, which leads to ECM deposition. As the blockade of the $P2Y_6$ receptor with MRS2578 suppressed the expression of ACE mRNA, and the blockade of $G\alpha_{12/13}$ suppressed the expression of TGF-β mRNAs, ATP and UDP work as an upstream regulator of the Ang II-TGF-β system. This also suggests that extracellular nucleotide-stimulated $G\alpha_{12/13}$ activity regulates the Ang II-TGF-β pathway through upregulation of ACE.

It has been reported that CTGF mediates some TGF-β-induced fibrogenic responses. Inhibition of CTGF synthesis or activity suppressed TGF-β-induced collagen synthesis (Perbal, 2004). It is also reported that CTGF synergizes fibrogenic responses with TGF-β by the mechanisms on the basis of the binding of CTGF to TGF-β or transcriptional suppression of Smad7 (Ruiz-Ortega *et al*, 2007). As CTGF expression was increased in a CA-$G\alpha_{13}$-Tg heart without affecting TGF-β expression, extracellular ATP and UDP may directly increase the expression of CTGF through $P2Y_6$ and $G\alpha_{12/13}$, with CTGF then promoting the production of TGF-β. Thus, extracellular nucleotides have an important function in fibrogenic responses of the heart.

Diastolic dysfunction associated with preserved systolic function is increasingly recognized as a critical cause of heart failure. As the cardiac ECM is the major determinant of myocardial stiffness during diastole, cardiac fibrosis contributes to diastolic dysfunction. We found that 6 weeks of TAC induces impairment of LV diastolic functions, which were attenuated by the inhibition of $G\alpha_{12/13}$ signalling or purinergic $P2Y_6$ receptors. As cardiac fibrosis associated with maladaptive hypertrophy is thought as a cause of impairment of cardiac function, purinergic receptors may be promising targets for the treatment of heart failure.

Materials and methods

Animals and TAC surgery

All protocols using mice and rats 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) and were approved by the guidelines of Kyushu University. Transgenic C57BL/6J mice expressing p115-RGS were tried to generate three times. We obtained only one line that was used in this study. Two lines of transgenic mice expressing CA-$G\alpha_{13}$ were generated (lines 1 and 5). Heterozygote of line 5 was used in this study. Age-matched male WT C57BL/6J mice were used as control. TAC surgery was performed on 8- to 10-week-old male p115-Tg and WT C57BL/6J mice. A mini-osmotic pump (Alzet) filled with saline, MRS2578, or suramin was implanted intraperitoneally 3 days after TAC into 6-week-old male C57BL/6J mice. Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Haemodynamic measurements and histological analyses

Transthoratic echocardiography was performed using ALOKA ultrasonic image analysing system (SSD-5500) equipped with 7.5 MHz imaging transducer. Blood pressure was monitored using tail-cuff system (BP-98A, Softron). LV pressure and heart rate were measured with a micronanometer catheter (Millar 1.4F, SPR 671, Millar Instruments). Histological analyses can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Isolation of cardiomyocytes and transfection

Cultures of neonatal rat cardiac myocytes and adenoviral infection were performed as described previously (Nishida *et al*, 2000). Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Pulldown assay and western blot analysis

Methods for pulldown assay and western blot analysis can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Measurement of extracellular nucleotides concentration

The determination of extracellular ATP concentration (2×10^5 cells per well) was performed using ATP Bioluminescence Assay Kit CLSII (Roche). The concentration of extracellular UDP in the supernatant of culture medium was analysed with an HPLC system (Jasco) as described previously (Koizumi *et al*, 2007). Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Measurement of mRNA expressions

Real-time RT–PCR was performed as described (Nagamatsu *et al*, 2006; Nishida *et al*, 2007). Sequences for PCR primers and Taqman probes were described in Supplementary information (Supplementary Table 5). The PCR primers used for expression analysis of P2Y receptors are described in Supplementary Table 6. Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Statistical analysis

Data were shown as means ± s.e.m. Statistical comparisons were made with two-tailed Student's *t*-test or analysis of variance followed by Student–Newman–Keuls procedure, with significance imparted at *P*-values <0.05.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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RESEARCH ARTICLE

A Human Phospholamban Promoter Polymorphism in Dilated Cardiomyopathy Alters Transcriptional Regulation by Glucocorticoids

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Depressed calcium handling by the sarcoplasmic reticulum (SR) Ca-ATPase and its regulator phospholamban (PLN) is a key characteristic of human and experimental heart failure. Accumulating evidence indicates that increases in the relative levels of PLN to Ca-ATPase in failing hearts and resulting inhibition of Ca sequestration during diastole, impairs contractility. Here, we identified a genetic variant in the PLN promoter region, which increases its expression and may serve as a genetic modifier in dilated cardiomyopathy (DCM). The variant AF177763.1:g.203A>C (at position −36bp relative to the PLN transcriptional start site) was found only in the heterozygous form in 1 out of 296 normal subjects and in 22 out of 381 cardiomyopathy patients (heart failure at age of 18–44 years, ejection fraction = 22 ± 9%). In vitro analysis, using luciferase as a reporter gene in rat neonatal cardiomyocytes, indicated that the PLN-variant increased activity by 24% compared to the wild type. Furthermore, the g.203A>C substitution altered the specific sequence of the steroid receptor for the glucocorticoid nuclear receptor (GR)/transcription factor in the PLN promoter, resulting in enhanced binding to the mutated DNA site. These findings suggest that the g.203A>C genetic variant in the human PLN promoter may contribute to depressed contractility and accelerate functional deterioration in heart failure. Hum Mutat 0,1–8, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: promoter; polymorphism; transcriptional factor; GR; GRE; cardiomyopathy; PLN; SR Ca-ATPase

INTRODUCTION

Heart failure is a multifactorial syndrome in which intrinsic myocardial dysfunction contributes to cardiac dilation and diminished ejection performance, leading to progressive cardiac deterioration or sudden death [Richardson et al., 1996; Seidman and Seidman, 2001]. Genes causally associated with cardiomyopathy have been identified through nonbiased genetic analysis or by candidate gene studies in experimental system [Geisterfer-Lowrance et al., 1996; Franz et al., 2001]. Thus, molecular modifiers of heart failure include mutations of genes that encode cytoskeletal, sarcomeric, nuclear membrane, and calcium handling sarcoplasmic reticulum (SR) proteins. These findings have implicated pathogenic mechanisms whereby perturbation of structural integrity, contractile force dynamics, and calcium regulation within the cardiac myocyte intrinsically contribute to myocardial disease.

Abnormal calcium homeostasis is a prototypical mechanism for contractile dysfunction in failing cardiomyocytes. Depressed calcium cycling in experimental and human heart failure reflects, at least in part, impaired calcium sequestration by the SR [Chien, 2000; MacLennan and Kranias, 2003]. Calcium sequestration is mediated by a Ca-transport ATPase (SERCA2a), whose activity is modulated by alteration in the expression and phosphorylation of phospholamban (PLN; MIM# 172405) [Luo et al., 1996; Simmerman and Jones, 1998]. In experimental models, expression levels of PLN closely correlate with basal contractile parameters and their responses to β-agonists [Luo et al., 1994; Kadambi et al., 1996; Brittsan et al., 2000; Dash et al., 2001]. In human heart failure, the levels of PLN are increased relative to SERCA2a, resulting in higher inhibition of the Ca-pump's Ca-affinity, which impairs relaxation [Beuckelmann et al., 1992; Meyer et al., 1995; Hasenfuss, 1998]. As a double insult, the phosphorylation status of PLN is decreased, leading to increased inhibitory function and further depression of SR Ca-cycling. Thus, PLN is a major

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Ca-regulatory protein and efforts have concentrated on identifying naturally occurring mutations in the human PLN gene, which may perturb its activity and contribute to dilated cardiomyopathy (DCM). Indeed, three mutations in the coding region of the human PLN gene have been identified that are associated with familial cardiomyopathy [Haghighi et al., 2003, 2006; Schmitt et al., 2003]. However, parallel studies on genetic variants in the PLN promoter region, which may alter its expression levels, are limiting.

The PLN gene is located on human chromosome 6 [Fujii et al., 1991] and the 200 bp of its 5′ flanking region exhibits significant sequence homology between human, rabbit, rat, and mouse [Fujii et al., 1991; Haghighi et al., 1997; McTiernan et al., 1999a, 1999b]. Importantly, this segment of the 5′ upstream region of the human PLN gene contains conserved consensus motifs for GATA, CP1/NF-y, M-CAT, and E-box elements, which are also found in other mammalian species [Haghighi et al., 1997; McTiernan et al., 1999a]. However, the importance of these elements in regulation of PLN gene expression under physiological and pathophysiological conditions remains uninvestigated. Indeed, most studies indicate that cardiac PLN expression levels are maintained under stress and remodeling conditions, including pressure overload, hypertrophy, and failure [Ito et al., 2001; Kogler et al., 2003; Mills et al., 2006]. A recent study reported the presence of a rare human mutation in this highly conserved PLN promoter region (A>G at −77 bp), which was associated with increased PLN (1.5-fold) expression [Minamisawa et al., 2003]. This variant was found in 1 out of 87 hypertrophic cardiomyopathy patients, suggesting a role of the PLN promoter mutant in depressed Ca cycling, leading to hypertrophy.

In this study, we sought to identify naturally occurring PLN promoter mutations in nonfamilial heart failure patients. A novel point genetic variant (A>C) at position AF177763.1:g.203A>C (at −36bp relative to the PLN transcriptional start site: −36A>C) in the 5′ UTR region of the PLN gene was detected only in the heterozygous state in 22 heart failure patients and one normal subject. In vitro studies on the functional significance of this genetic variant revealed that it increases PLN expression levels by altering glucocorticoid nuclear receptor (GR)-mediated regulation of transcription.

MATERIALS AND METHODS

Mutation Identification

Informed consent was obtained from participating subjects. All protocols were approved by the institutional review board of the Onassis Cardiac Surgery Center (Athens, Greece) or the University of Cincinnati College of Medicine, (Cincinnati, OH). Genomic DNA was isolated either from whole blood or from paraffin blocks containing heart tissue. The genomic reference with GenBank accession number AF177763.1 was used to retrieve the PLN sequence corresponding to proximal promoter and exon 1. A 600-bp fragment of the PLN gene, containing the PLN promoter region was amplified by PCR, using 60 ng of genomic DNA and a high-fidelity *Taq* polymerase. The primers were: sense, 5′CTAAGCCTGAAGATGC3′ and antisense, 5′CCAGTAACCA GGATC3′, tagged with M13 forward and reverse primer sequences, respectively. The conditions were: one cycle at 94°C for 3 min, linked to 30 cycles at 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 53°C for 1 min, and 72°C for 10 min. The gel purified PLN DNA fragment was sequenced using automated dye-primer chemistry. The generated sequences were compared with the reported human PLN sequences by a computational method and the electropherograms were inspected individually for confirmation. The GenBank accession number AF177763.1 was used as a reference for numbering the PLN promoter polymorphism.

Echocardiography

Comprehensive 2D and Doppler echocardiography was performed according to the recommendations of the American Society of Echocardiography [Levy et al., 1990]. Left ventricular dimensions (interventricular septum end-diastolic thickness [IVEDT], left ventricular posterior wall end-diastolic thickness [PWEDT], left ventricular end-systolic and end-diastolic diameter [LVESD and LVEDD]) were measured with M-mode echocardiography, using the left parasternal window. Left ventricular volumes and ejection fraction (LVEF) were determined by apical two- and four-chamber views using the modified Simpson rule [Levy et al., 1990].

Cloning of the Human PLN Gene Promoter-Reporter Constructs

A PCR-based strategy was employed using high-fidelity DNA polymerase to amplify the mutant region from human PLN genomic DNA, comprising the upstream PLN promoter. A 510-bp DNA fragment was PCR-amplified from normal and DCM genomic DNA utilizing the primers 5′-TACCTGTGTTTATTTTTCTC-3′ and 5′-AAGAAGAATTACCAAAGTCAGC3′. To facilitate cloning, Kpn I and Xho I sites were added to the beginning of the primers. The 510-bp fragment containing the engineered Kpn I and Xho I sites was subcloned into the pBlueScript vector (Stratagene, La Jolla, CA). The upstream PLN promoter region was verified by DNA sequence analysis. Then, the PLN promoter fragment containing either the nucleotide transition, −36A>C, or the wild type sequences of the PLN gene was digested with Sac I and Pst I, and cloned into pGL3-Basic (Promega, Madison, WI) to create the PLN promoter-luciferase reporter constructs.

About 600 bp of 5′ upstream of the PLN gene sequences were scanned for putative transcription factor binding sites, using public domain software (Transcription Element Search Software; www.cbil.upenn.edu/tess; TFBLAST of TRANSFAC 6.0; Biobase Corporation, Beverly, MA; www.gene-regulation.com/cgi-bin/pub/programs/tfblast/tfblast.cgi).

Cardiomyocyte Culture, Transient Transfection, and Luciferase Assays

Ventricular myocytes were isolated from 1-day-old Sprague-Dawley rats and cultured as described [Minamisawa et al., 2003]. For promoter-reporter studies, after 24 hr incubation with serum-free medium, the myocytes were transiently cotransfected with 300 ng of each PLN luciferase test plasmid and 75 ng of phRL-TK control plasmid (Promega). The cells were harvested in Passive Lysis Buffer (Promega) 48 hr after transfection, and were stored at −80°C until processed for the luciferase assay. The cells were allowed to grow in the absence or presence of 3 μM dexamethasone for the last 45 hr of the 48-hr incubation period. Luciferase assays were performed according to the protocol of the Dual Luciferase Assay System (Promega). Each data point represents the mean and the standard error of the mean (SEM) of seven experiments.

Electrophoretic Mobility Shift Assays

Nuclear extracts from ventricular tissue samples were prepared as described previously [Brown et al., 2005] with modifications. Briefly, ventricular tissue was pulverized at liquid N_2 temperatures, homogenized at low speed in buffer containing 10 mM HEPES

(pH 9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 25 μg/ml leupeptin, 0.2 mM sodium orthovanadate, and 0.1% (vol/vol) Triton X, then vortexed and incubated on ice for 10 min. After centrifugation (5,000 *g* for 10 min), the pellet was suspended in solution containing 20 mM HEPES (pH 7.9), 25% (vol/vol) glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethanesulphonylfluoride (PMSF), 0.5 mM DTT, 25 μg/ml leupeptin, and 0.2 mM sodium orthovanadate, and then vortexed. This suspension was incubated on ice for 40 min with rigorous vortexing every 10 min. After centrifugation (10,000 *g* for 15 min), the supernatant was retained as a crude nuclear extract. Protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay with bovine serum albumin as a standard.

A double-stranded 20-bp oligodeoxynucleotide, containing the PLN promoter wild type (5′-CCTCCCTAG{A}{ACACTTTTTC-3′; underlined, glucocorticoid binding element) or mutant form (5′-CCTCCCTAG{C}{ACACTTTTTC-3′; bold, mutated nucleotide) was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega), and was purified using a G-50 Sephadex column (Amersham Pharmacia Biotech, Piscataway, NJ). The binding reactions were performed in a final volume of 10 μl that contained 20 μg of nuclear protein, 10 mM Tris · HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (vol/vol), and 1 μg of poly(dI-dC). After a 10-min preincubation at room temperature, the labeled probe (1×10^5 cpm/reaction) was added to each reaction and the reactions were incubated for an additional 20 min at room temperature. The DNA-protein complexes were separated on 6% nondenaturing polyacrylamide gels in 1 × Tris borate-EDTA buffer. Gels were vacuum-dried and exposed to X-ray film at −20°C, using intensifying screens. Competition assays with 100-fold molar excess of unlabeled consensus oligodeoxynucleotide or control nonspecific oligodeoxynucleotide were performed to ensure that the signal was specific. The commercially available oligonucleotide containing the common glucocorticoid consensus, 5′-GACGGTACAAAATGTTCTAGG-3′ (Active Motif, Carlsbad, CA) and antiglucocorticoid antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for specific binding activity confirmation. A double-stranded 22-bp oligodeoxynucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a consensus nuclear factor-κB (NF-κB) binding site (underlined) was used as positive control.

Statistics

Data are presented as mean ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. A P value of <0.05 was considered statistically significant. The agreement with the Hardy-Weinberg expectations (HWE) of genotype frequencies was determined using the chi-squared test based on the number of observed and expected heterozygotes and the exact test based on the number of observed and expected genotypes [Guo and Thompson, 1992].

RESULTS

Clinical History

A total of 381 DCM patients and 296 normal subjects without any known cardiomyopathy history were recruited from the University Hospital, Cincinnati Heart Failure/Transplant Program (Cincinnati, OH) and the Onassis Cardiac Surgery Center (Athens, Greece). The clinical characteristics and the demographic data for the DCM populations are summarized in Table 1.

TABLE 1. Clinical Characteristics of the United States and Greek DCM Patients With Heart Failure

	Ethnicity	
	United States	Greek
n	163	218
Age (years)	44.95 ± 3.3	40 ± 6.2
Gender (%)		
Male	72	82
Female	28	18
Etiology (%)		
Dilated cardiomyopathy	94.27	100
Ischemic cardiomyopathy	5.76	—
Functional class (% NYHA III/IV)	67.2	70.6
LVEF (%)	23.6 ± 8	26.4 ± 6

DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association Classification.

Comorbid conditions in the cohorts included: hypertension (8%), diabetes (6%), hypercholesterolemia (12%), and atrial fibrillation (12%). The medications used by the DCM patients were angiotensin-converting enzyme (ACE) inhibitors (97%), diuretics (94%), digoxin (98%), beta blockers (75%), Ca-channel blockers (12%), and antiarrhythmics (45%).

Identification of a Genetic Variant in the Human PLN Promoter Region

In the initial discovery study, the PLN gene promoter region 600 bp upstream from the transcriptional start site [McTiernan et al., 1999a] was sequenced in 40 unrelated Greek DCM patients. The sequencing of this region identified a single nucleotide transition from A>C at position AF177763.1:g.203A>C (at −36bp relative to the PLN transcriptional start site: −36A>C) (Fig. 1A). We subsequently screened an additional 178 Greek DCM patients to determine the frequency of this PLN genetic variant. The −36A>C substitution was found in another 15 patients (16/218 total) and it was only present in the heterozygous form, reflecting an allelic frequency of 3.66% in the Greek DCM population. To confirm the presence of this novel PLN promoter variant in a different heart failure population, 163 Caucasian DCM patients (University Hospital, Cincinnati Heart Failure/Transplant Program, University of Cincinnati, OH) were also screened. The −36A>C variant was found in the heterozygous form in six patients, reflecting an allelic frequency of 1.84%. The characteristics of the patients with the identified transition in the PLN gene in the two cohorts were similar (Table 2). The PLN −36A>C variant carriers presented with heart failure symptoms and were diagnosed with cardiomyopathy at ages ranging from 18 to 44 years. Echocardiography studies indicated severe left ventricular dilatation and systolic dysfunction (e.g., ejection fraction of 22 ± 9%). Their symptoms remained under control with drug treatments. However, some patients' symptoms progressively deteriorated (New York Heart Association [NYHA] Classification, NYHA class III), leading to the death of one patient at the age of 48 years and heart transplantation in another patient at the age of 46 years. The promoter variant −36A>C was found in only 1 normal control subject out of 296 screened. There were no departures from Hardy-Weinberg equilibrium for allelic frequencies in either DCM or control populations.

The promoter region of the human PLN gene, containing the genetic variant is a highly conserved region among species (Fig. 1B) [McTiernan et al., 1999a]. Therefore, it was hypothesized that this change in nucleotide sequence might alter PLN

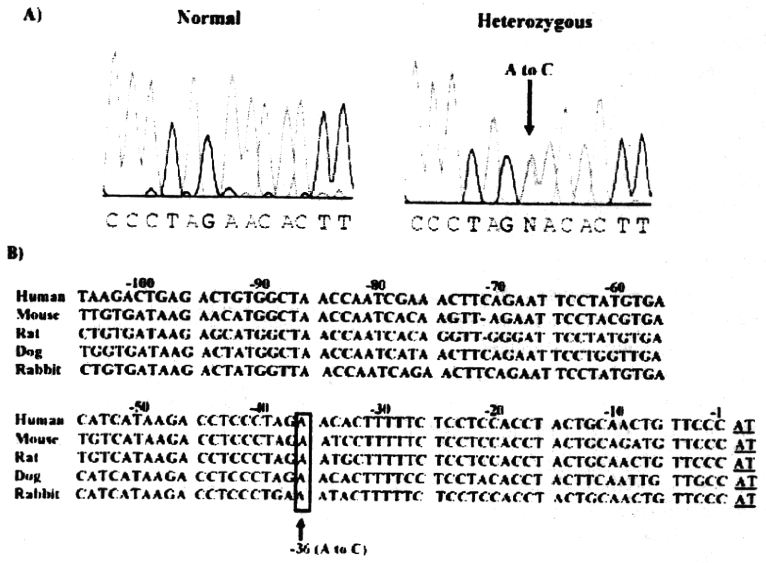

FIGURE 1. **Genomic DNA sequence analysis of the PLN promoter region. A: Partial nucleotide sequences of the PLN promoter region in normal subjects and DCM patients heterozygous for the AF177763.1:g.203A>C (at −36 bp from transcriptional start site: −36A>C) substitution. B: Sequence comparison of the proximal mammalian PLN promoter sequences was performed by the FASTA program (http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml) (GenBank reference sequence numbers AF177763.1, AF037348.1, L03381.1, and M63600.1). The numbers correspond to human nucleotides upstream of exon 1 (transcription start site, underlined). The position of the A>C transition (boxed) is indicated. Gaps are shown by dash. Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence corresponding to proximal promoter and exon 1 and the transcription start site as a reference. [Color figure can be viewed in the online issue, which is available at http://www.interscience.wiley.com.]**

TABLE 2. **Clinical Characteristics of Dilated Cardiomyopathy Patients With the −36A>C Substitution in the PLN Gene***

	Wild-type allele	Mutant allele
n	359	22
Age (years)	42±3.1	40±6
Etiology (%)		
Dilated cardiomyopathy	94.27	100
Functional class (% NYHA III/IV)	67.2	66.66
LVEF (%)	24.5±8	22±9

*Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence, corresponding to proximal promoter and exon 1 as a reference.
DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association Classification.

promoter activity and consequently its regulation of SERCA2a, and thus contribute to the pathophysiology of heart failure.

In Vitro Assays of PLN Promoter Activity

To determine the potential functional importance of the identified genetic variation on PLN transcriptional regulation, we generated reporter constructs that expressed luciferase under the control of the putative promoter sequences from the human PLN gene. When neonatal rat cardiomyocytes were transiently transfected with luciferase reporters under the control of wild-type (PLN-WT) or "mutant" PLN (PLN-MT) promoters, the −36A>C transition resulted in a significant increase of 24% in transcriptional activity, compared to the wild-type promoter (Fig. 2A). To examine whether the −36A>C point transition in the PLN gene may alter regulation by any of the sequence-specific DNA-binding proteins, such as transcription factors, we performed a computer sequence search for putative regulatory binding sites. We identified a potential sequence for the glucocorticoid response element (GRE) within the mouse PLN promoter (Fig. 2B). Our DNA scanning revealed that the −36A>C substitution was within the putative glucocorticoid receptor binding site of the PLN promoter gene. To further investigate the regulation of PLN gene expression by the glucocorticoid response element, the luciferase reporter constructs of PLN-WT and PLN-MT were transiently transfected into rat neonatal cardiac cells in the absence or presence of dexamethasone. The induced luciferase activity of PLN-WT was significantly increased when dexamethasone was present, while there was no effect of dexamethasone on the PLN-MT, compared to basal levels (Fig. 2A). The lack of luciferase activity induction in PLN-MT following stimulation of transfected cells by dexamethasone may indicate that the genetic variant abolished the direct or indirect mediation of the dexamethasone-mediated enhancement of the reporter gene activity.

To further examine the functional significance of the −36A>C PLN promoter variant, gel mobility shift assays were employed. Using nuclear extracts from mouse heart, the binding assays showed that both synthetic WT (Fig. 3; lanes 13 and 14) and MT (Fig. 3; lanes 11 and 12) oligonucleotides were able to form a DNA-protein complex, indicating transcription factor binding. However, stronger binding was observed with the PLN-MT oligonucleotide, demonstrating that this sequence has a higher affinity for transcription factor binding. Binding was completely blocked in the presence of 100-fold excess of the cold-labeled WT (Fig. 3; lanes 3 and 4) or MT oligonucleotide (Fig. 3; lanes 5 and 6), used as specific competitors. Nuclear lysate was used as a negative control and it did not form any complexes in the presence of either synthetic WT or MT oligonucleotide (Fig. 3; lanes 7–10). However, an oligonucleotide containing a consensus NF-kB binding site, used as a positive control for nuclear lysate activity, yielded DNA-protein complexes in the lysates. These findings

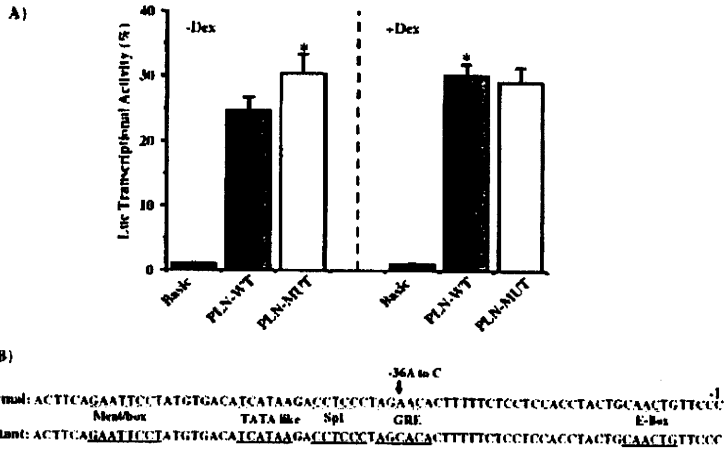

FIGURE 2. Effect of the –36A > C genetic variant on human PLN promoter activity. **A:** Rat neonatal cardiomyocytes were transiently transfected with a luciferase expression vector driven by PLN-WT or PLN-MT (–36A > C) promoters, and were cultured in the absence (left) or presence (right) of 3 μM dexamethasone (Dex) for 45 hr. Transcriptional activity of the promoters was defined as a ratio of firefly luciferase activity to Renilla luciferase activity in the same cells, and normalized to the mean basal transcriptional activity of the promoter-less pGL3-basic vector. **B:** Sequence alignment of the normal and mutant human PLN upstream promoter regions. The relative positions of the promoter starting site (-1) and of the potential regulatory sequences (underlined) are indicated. The values are expressed as means ± SEM (n = 7). *P < 0.05 vs. PLN-WT without Dex (two-way ANOVA and Student-Neuman-Keuls test). Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence corresponding to proximal promoter and exon 1 as a reference.

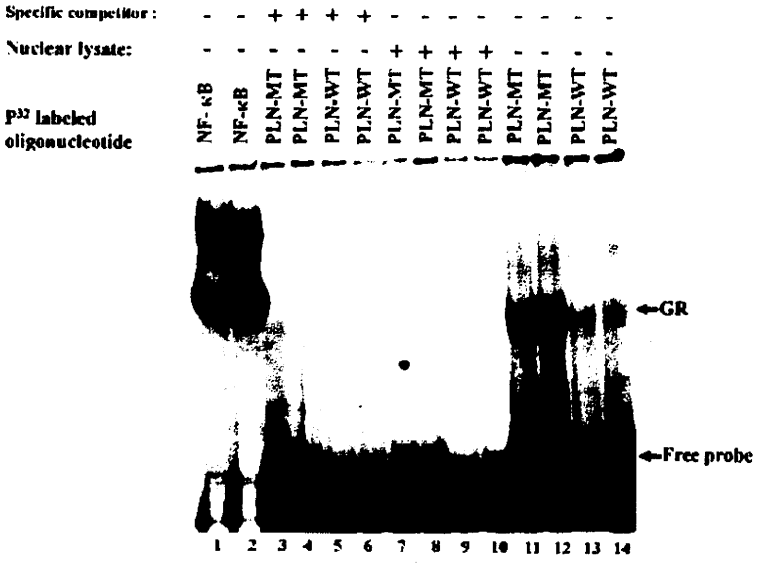

FIGURE 3. Electrophoretic mobility gel shift assay of wild-type and genetically-altered glucocorticoid elements in the PLN promoter sequences. Electrophoretic mobility gel shift assays were used to determine DNA–protein complex formation using nuclear extracts from mouse hearts. NF-kB was used as a positive technical control (lanes 1 and 2); nonlabeled wild-type (PLN-WT, lanes 3 and 4) and altered (PLN-MT, lanes 5 and 6) were used as specific competitors; and nuclear lysate as a negative control (lanes 7–10); PLN-MT oligonucleotide (lanes 11 and 12) and PLN-WT oligonucleotide (lanes 13 and 14). Duplicate samples were assayed for each treatment.

suggest that the quality of the nuclear lysates and the binding conditions were appropriate (Fig. 3; lanes 1 and 2).

To verify whether the binding activity from heart nuclear extracts reflects a specific interaction between the GR with the PLN-WT and PLN-MT probes, as predicted based upon computer searches, we employed a commercially available oligonucleotide, containing a known consensus GRE sequence. This oligonucleotide was used in DNA binding and competition studies, designed to assess specificity of our DNA-protein complexes. The GRE consensus oligonucleotide displayed a strong DNA-protein binding complex in nuclear extracts (Fig. 4; lane 3). Furthermore, this commercially available oligonucleotide could completely block DNA-protein complex formation with PLN-WT, PLN-MT, and the GRE oligonucleotide in the nuclear extracts (100-fold excess; Fig. 4; lanes 4–6). The consensus GRE containing oligonucleotide and the PLN promoter-derived sequences (PLN-WT and PLN-MT) demonstrated identical migration of the DNA-protein complexes (Figs. 3 and 4). It was interesting to note that the

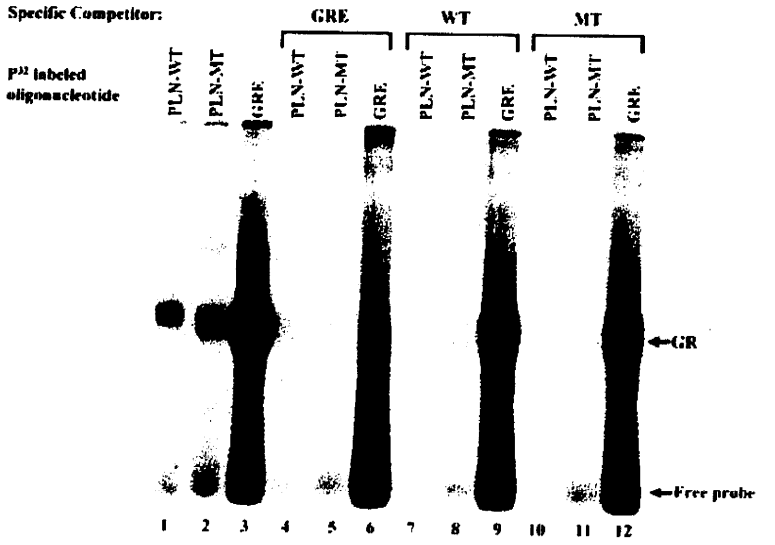

FIGURE 4. **Electrophoretic mobility gel shift assay of the PLN wild-type and genetically-altered glucocorticoid element specificity in the presence of common glucocorticoid consensus. Consensus glucocorticoid receptor element (GRE), PLN-WT, and PLN-MT motifs were used to examine binding specificity in cardiac nuclear extracts from wild-type mice. Lanes 1–3: cardiac nuclear extracts reacted with radiolabeled oligonucleotides of PLN-WT, PLN-MT, and consensus GRE sequences. Lanes 4–6: consensus GRE oligonucleotides were used as specific competitor (100 × unlabeled GRE oligonucleotides). Lanes 7–9 and lanes 10–12: PLN-WT and PLN-MT oligonucleotides were used as competitors (100 × -fold unlabeled oligonucleotide), respectively.**

PLN-WT or PLN-MT oligonucleotides could not completely compete with the common glucocorticoid consensus (Fig. 4; lanes 9 and 12). Taken together, these results indicate that the GR binds specifically to the PLN-WT and PLN-MT promoter sequences, albeit at lower affinity than the consensus GRE employed.

DISCUSSION

In this study, we identified a novel variant (–36A>C) in the human PLN promoter region in 22 heart failure patients and one normal subject, which appears to enhance promoter activity and alter the GR receptor binding element. Importantly, this PLN promoter variant was identified in two heart failure populations. The allelic frequencies in two ethnic populations and in controls were in Hardy-Weinberg equilibrium, indicating that this genetic variant is heritable and a combination of the –36A>C PLN variant with other genetic and environmental modifies may contribute to the time course of the disease in the patients. The identified nucleotide substitution is in close proximity to the putative TATA (5′-TCATAA-3′) boxes at position –48 to –53 in an evolutionarily conserved PLN gene region between species, and may play a significant role in regulating PLN gene expression. Indeed, in vitro studies of this genetic variant indicated that it may increase PLN expression levels and consequently, depress SR Ca cycling associated with cardiomyopathy. The functional significance of increased PLN levels in cardiac muscle has previously been demonstrated through the generation and characterization of transgenic mouse models [Kadambi et al., 1996; Dash et al., 2001]. Consistent with findings in transgenic mice, an increase in the apparent stoichiometry of PLN to SERCA2a, as a result of the PLN promoter genetic variant, may contribute to the depressed Ca cycling and deterioration of cardiac function.

Recently, there has been a considerable upsurge of interest in the influence of *cis*-acting genetic variations on gene transcription. Furthermore, these mutations and polymorphisms, found in various gene promoter regions, have been reported to affect gene expression and impact function [Collins et al., 2003; Hudson, 2003; Buckland et al., 2004; Guy et al., 2004; Schulz et al., 2006]. Importantly, the PLN promoter variant (A>C, underlined below), identified herein, was within a novel consensus sequence segment that matched a glucocorticoid receptor-binding site (5′-AGAA-GA-3′). Previous studies have shown that thyroid hormone and glucocorticoids regulate the expression of several genes, including calcium cycling proteins [Kiss et al., 1994, 1998; Smith and Smith, 1994; Brittsan et al., 1999; Muangmingsuk et al., 2000]. Thyroid hormone was reported to mediate changes in PLN protein levels [Kiss et al., 1994, 1998; Brittsan et al., 1999] possibly through interaction with thyroid hormone elements residing in the PLN promoter region. Glucocorticoids downregulate Na-Ca exchanger mRNA levels and activity in aortic myocytes [Smith and Smith, 1994], while they increase expression of alpha-myosin heavy chain (MHC) and decreased expression of beta-MHC in neonatal rat cardiomyocytes [Muangmingsuk et al., 2000]. These changes suggest that, similar to thyroid hormone-mediated transcriptional activation, the glucocorticoid effects may also be mediated in part through transcriptional mechanisms. Indeed, the level of PLN transcripts was significantly decreased, when rat neonatal cardiomyocytes were treated with cytokines (interleukin [IL]-1β, tumor necrosis factor [TNF]) [McTiernan et al., 1997], while dexamethasone significantly elevated the levels of PLN transcripts [McTiernan et al., 1997], indicating the direct effects of dexamethasone on PLN gene regulation. In this report, similar results were obtained with dexamethasone induction of PLN-WT promoter expression. In contrast, dexamethasone did not increase the luciferase transcriptional activity of the PLN-MT promoter, suggesting that the –36A>C substitution may have abolished the interaction site for glucocorticoid receptor elements in the PLN gene.

The role of transacting elements in the transcriptional activity of the PLN gene remains poorly understood and the nuclear proteins involved in the regulation of the gene through binding to these elements are unknown. Our previous studies on

characterization of the mouse PLN promoter indicated that 200 bp proximal to the transcriptional initiation site is sufficient for moderate (40%) expression of PLN levels [Haghighi et al., 1997]. The dexamethasone-responsive PLN gene sequences are located within the 200-bp proximal promoter region of the mouse and human PLN gene, which are highly conserved between species [Haghighi et al., 1997; McTiernan et al., 1999a]. Increased luciferase activity in the promoter-reporter studies suggest that GREs within this region may contribute to the modulation of transcriptional regulation via DNA–protein interactions of the PLN gene as further supported by electrophoretic mobility gel shift assay studies. Obviously, the limitation of this study is that the upregulation of the PLN promoter activity presented here is primarily from in vitro studies; in vivo relevance of these finding could not be performed due to lack of cardiac biopsies from affected individuals.

The glucocorticoid receptor is a ligand-dependent transcription factor with both hormone and DNA binding domains, affecting the transcription of specific genes [Schoneveld et al., 2004]. Specifically, glucocorticoid hormones are the major mediators of systemic stress responses [Brent et al., 1991] and it has been suggested that they may regulate SR function and cellular calcium homeostasis in the myocardium [Rao et al., 2001; Aoyama et al., 2005]. The possible mechanisms may involve modulation of PLN phosphorylation through Ca/calmodulin-dependent protein kinase II (CaM kinase II) [Rao et al., 2001]. Interestingly, the CaM kinase II dependent phosphorylation site of PLN, Thr17, has been implicated in stress responses of the cardiomyocytes [Hagemann et al., 2000; Zhao et al., 2004]. Therefore, under stress conditions, GR modulation of PLN activity and/or expression levels may influence SR Ca cycling and myocardial function, which may be beneficial during early cardiac remodeling but deleterious under pathophysiological conditions. However, the abolished PLN GRE site by the −36A>C genetic variant eliminates the GR-mediated regulation, resulting in chronic increases in PLN expression levels and inhibition of SERCA activity, which may accelerate deterioration of function in DCM.

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