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MOL 786

Down-regulation of Na^+/Ca^{2+} exchanger by fluvastatin in rat cardiomyoblast H9c2 cells; involvement of RhoB in Na^+/Ca^{2+} Exchanger mRNA stability

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Running title: NCX1 down-regulation by fluvastatin in H9c2 cells.

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Discussion=1138.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; Mev, mevalonic acid; Flv, fluvastatin, LPC, Lysophosphatidylcholine; C3, Clostridium botulinum C3 exoenzyme; ROCK, Rho-associated coiled-coil-forming protein kinase; NCX1, Na^+/Ca^{2+} exchanger 1; Y-27632, (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)- cyclohexanecarboxamide,

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Abstract: We investigated the effect of fluvastatin (Flv), a 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitor, on Na^+/Ca^{2+} exchanger 1 (NCX1) expression in H9c2 cardiomyoblasts. RT-PCR analyses revealed that Flv decreased NCX1 mRNA in a concentration- and time-dependent manner and NCX1 protein. This effect of Flv was due to inhibition of HMG-CoA reductase, because Flv failed to affect the NCX1 mRNA in the presence of mevalonate. Flv-induced down-regulation of NCX1 mRNA was also cancelled by farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), suggesting an involvement of small G-proteins. However, neither overexpression of constitutive active RhoA nor Ras affected NCX1 mRNA. In contrast, intracellular expression of C3 toxin, a specific inhibitor of Rho family proteins, decreased NCX1 mRNA, suggesting that Flv decreases NCX1 mRNA by inhibiting a signaling pathway of Rho family proteins other than RhoA. Conversely, lisophosphatidylcholine (LPC), an activator of Rho-signaling, increased both NCX1 mRNA and protein in a C3 toxin-sensitive manner. Western blot analyses revealed that membrane associated RhoB, which is isoprenylated either by FPP or GGPP, was decreased by Flv, but increased by LPC. Selective inhibition of gene expression by short interfering RNA duplex

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showed that RhoB but not RhoA is involved in the regulation of NCX1 mRNA and protein. When transcription was blocked by 5,6-dichlorobenzimidazole riboside, the NCX1 mRNA stability was decreased by Flv. Chronic treatment of rat with Flv *in vivo* also down-regulated the cardiac NCX1 mRNA. These results suggest that a RhoB-mediated signaling pathway regulates cardiac NCX1 levels by controlling the NCX1 mRNA stability. (247 words)

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The Na^+/Ca^{2+} exchanger (NCX) plays a pivotal role in the regulation of intracellular Ca^{2+} homeostasis in cardiac myocytes . There are three types of mammalian NCX. NCX1 is expressed ubiquitously but most abundantly in the heart, while NCX2 and NCX3 are expressed in the brain and in skeletal muscle (Yao et al., 1998). Several studies have shown that the expression of NCX1 mRNA is up-regulated in animal models of cardiac hypertrophy and heart failure (Pogwizd et al., 1999), as well as in human end-stage heart failure (Hasenfuss et al., 1999). On the other hand, NCX1 expression was down-regulated in some other heart failure models (Yao et al., 1998). However, the mechanism regulating NCX gene expression is still unknown.

HMG-CoA reductase inhibitors, known as statins, are widely used clinically to prevent coronary heart disease and systemic atherosclerosis. It is generally assumed that the beneficial effects of statins result from the inhibition of cholesterol synthesis (Goldstein and Brown, 1990; Levine et al., 1995). However, by inhibiting HMG-CoA reductase and thereby mevalonic acid (Mev) synthesis, statins also have other effects through prevention of the synthesis of various isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Grunler, et al. 1994), both of which are important lipid attachments for the post-translational modification of a

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variety of small GTP-binding proteins, such as Ras and Rho GTPases (Casey, 1995). Ras and Rho family proteins coordinately regulate various cellular processes such as differentiation, proliferation and apoptosis (Scita et al., 2000; Van Aelst and D'Souza-Schorey, 1997).

We postulated that fluvastatin (Flv), one of the lipophilic statins, may modulate NCX1 mRNA expression in cardiac myocytes by affecting small GTP-binding proteins. In the present study, we investigated the effects of Flv on NCX1 mRNA levels in H9c2 cardiomyoblasts.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Grand Island, NY, USA). Flv was a gift from Novartis (Basel, Switzerland). The Rho kinase inhibitor Y27632 was provided by Welfide (Osaka, Japan). Mev lacton, FPP, GGPP, L-α-lysophosphatidylcholine, palmitoyl (LPC) and 5,6-dichlorobenzimidazol riboside (DRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. H9c2 cells were cultured in DMEM supplemented with 10 % fetal bovine serum and maintained at 37 ^{o}C in a humidified atmosphere of

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5 % CO_2/95 % air. Cells were plated on 35mm dishes at 10^4cells/cm^2.

RT-PCR. Total RNA was extracted from H9c2 cells by the acid-guanidine thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). First strand cDNA primed by random hexamers was prepared from total RNA (1 μg) using Moloney murine leukemia virus reverse transcriptase in a final reaction volume of 20 μl. The cDNA was diluted 5-fold with water, and used as a template for PCR analysis. Primers used to amplify NCX1 and glyceraldehyde-3-phosphate dehydrogenase (GPDH) mRNAs were designed based on the published cDNA sequences as described previously (Watanabe et al. 2002). The primers for RhoA and RhoB were GTG GTA AGA CAT GCT TGC TC (sense, 47-66) and GAT GAT GGG CAC ATT TGG AC (antisense, 339-320), and ATG TGC TTC TCG GTA GAC AG (sense, 526-545) and AGA AAA GGA CGC TCA GGA AC (antisense, 1133-1114), respectively (GenBank accession number: AY026068, NM022542). PCR was carried out with different amplification cycles as described previously (Ohkubo *et al.*, 2000). The predicted lengths of PCR products for NCX1, GPDH, RhoA and RhoB are 302, 500, 293 and 608 bp, respectively. The PCR products were separated by 1.5 % agarose gel electrophoresis and visualized by ethidium

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bromide staining. The images were analyzed using NIH image software. To quantitate mRNA levels, we first determined optimal PCR conditions with different amplification cycles. With our experimental conditions, PCR products of NCX1 and GPDH with control H9c2 cells were increased during 22-28 and 16-22 cycles, respectively. Therefore we used 24 and 26 amplification cycles for NCX1, and 18 and 20 cycles for GPDH. Under these conditions, the amounts of PCR products correlated well with the mRNA levels (0.1-5 μg) analyzed. In some experiments, mRNA levels were quantified using a real-time PCR system (Light cycler, Roche, Penzberg, Germany) with LightCyclar FastStart DNA Master SYBR Green I Kit. All results shown by semi-quantitative RT-PCR analysis were confirmed by the real-time PCR system.

Adenovirus infection. Recombinant adenovirus pAdTrack-CMV, pAdTrack-CMV containing cDNA encoding C3 toxin, constitutive active mutant of ras (rasV12) and constitutive active mutant of RhoA (RhoAV14) were prepared as described previously (Tashiro et al, 2003; Arai et al., 2003). Twenty four hours before infection H9C2 cells were plated on collagen-coated 50 ml (25 cm^2) culture flask at 5×10^5 cells/ cm^2. Adenovirus

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infection was done for 2 hours at a multiplicity of infection of 100 in 0.5 ml of culture medium. The cells were then cultured for 24 hours in DMEM medium containing 10% fetal bovine serum. Under these conditions, almost 100% of the cells expressed recombinant proteins as determined by visualization of GFP. Cells were then treated with Flv or LPC for additional 24 hours. RNA was extracted and mRNA levels of NCX1 and GPDH were determined by RT-PCR as described above.

Western blotting. H9C2 cells were incubated with or without flv or LPC for 24 h and then washed twice with phosphate buffered saline. Cells were then harvested with lysis buffer (10 mM Tris-HCl, pH7.4, 2mM EDTA, 1mM DTT, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). Whole cell lysates were sonicated and centrifuged at 500*g* for 5 minutes at 4 °C. The supernatants (12.5 μg protein) were subjected to Western blot analysis using anti-NCX1 rabbit polyclonal antibody as described previously (Iwamoto et al. 1998). For measurement of RhoB protein, whole cell lysates after removing nuclei were centrifuged at 25000*g* for 10 minutes at 4 °C. The supernatant was collected and used as cytosolic fraction. The pellet was washed twice with the lysis buffer by

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centrifuging at 25000*g* for 10 minutes at 4 °C. The pellet was resuspended with the lysis buffer and used as crude membrane fraction. Membrane and cytosolic fractions were then subjected to Western blot analysis using anti-RhoB rabbit polyclonal antibody (sc-180, Santa Cruz Biotechnology, Inc. California). The intensities of immunoreactive protein bands were analyzed using NIH Image software.

siRNA Reagents and Transfection. siRNA sequences was selected by siDirect (RNAi. Tokyo, Japan), that is a web-based online software system for selecting highly effective siRNA sequences (Naito et al., 2004). RNA oligonucleotides were synthesized by Proligo. Sequence CUA UCG AUC GGA CGU CGU ACG, was used as a control siRNA. siRNA against RhoA and RhoB corresponded to coding regions +255-270 and +1005-1026, respectively (GenBank accession number: AY026068, NM022542). H9c2 cells were plated in 6-cm culture dishes at a density of 4×10^4 cells/dish. Transfection of siRNA (100 pmol/well) was carried out using Polyfect (Qiagen) according to the manufacturer's protocol. After transfection, cells were cultured for 24-72 hours and subjected to RNA extraction and Western blot analysis. Changes in mRNA levels of NCX1, RhoA, RhoB and GPDH

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were determined by a real-time PCR system and semi-quantitative RT-PCR analysis as described above. Changes in protein levels of NCX1 and RhoB were determined by Western blot analysis as described above.

Treatment of rat with Flv *in vivo*. Six-week-old male Wister rats weighing 107-123g were divided into two groups (control and Flv groups). The rat was housed individually in one cage, and fed a grained diet which was daily given about 10 % of body weight. Flv group were fed the same diet but containing 1mg/g Flv. Flv group took approximately 60 mg Flv/kg/day. After one week, rats were sacrificed and total RNA was extracted from heart as described above. NCX1 mRNA levels were examined by RT-PCR using the real-time PCR system.

Data Analysis. Statistical analyses of the data were performed by the paired Student's *t* tests for two data comparison and one-way analysis of variance with the Dunnett two-tailed test for multiple data comparison. *P* values of less than 0.05 were considered to be statistically significant.

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Results

Effect of Flv on NCX1 mRNA expression

H9c2 cells were incubated for various time periods with 5 μM Flv and NCX1 mRNA levels were evaluated by semi-quantitative RT-PCR. As shown in Figure 1A and B, Flv decreased the NCX1 mRNA in a time dependent manner. Exposure to Flv for 24 h decreased NCX1 mRNA to 57 % of the control (Fig.1B). This effect of Flv was concentration-dependent and the minimum effective concentration was around 0.5 μM (Fig.1C).

Role of HMG-CoA reductase pathway in the regulation of NCX1 mRNA level

Because HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, we determined whether the addition of mevalonate would prevent the inhibitory effect of Flv on the NCX1 mRNA. H9c2 cells were incubated with 5 μM Flv for 24 hours in the absence or presence of 100 μM Mev. Mev prevented the decrease in the NCX1 mRNA caused by Flv (Fig 2), suggesting that the effect of Flv was due to inhibition of HMG-CoA reductase.

FPP and GGPP are downstream metabolites of Mev and are essential for isoprenylation of small G proteins. Statins are known to prevent

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isoprenylation of small G proteins, thereby inhibiting the small G protein-mediated signaling pathway. Treatment of H9c2 cells with Flv (5 μM) in the presence of FPP (2.5 μM) or GGPP (2.5 μM) for 24 hours prevented the decrease in NCX1 mRNA induced by Flv (Fig. 2). These results suggest that the effect of Flv on the level of NCX1 mRNA resulted from a decrease in intracellular levels of FPP or GGPP and thus it is likely that small G proteins are involved in regulating the level of NCX1 mRNA.

Involvement of small G-protein signaling pathway in the regulation of NCX1 mRNA level

Because geranylgeranylation and farnesylation is required for activation of small G-proteins, we examined the effect of expression of constitutive active mutant of RhoA, RhoAV14 and that of Ras, RasV12. Infection of recombinant adenovirus encoding RhoAV14 or RasV12 did not alter NCX1 mRNA. In contrast, expression of botulinum C3 exoenzyme, a specific inhibitor of Rho family protein (Paterson et al., 1990; Ridley and Hall, 1992; Sekine et al., 1989) significantly decreased NCX1 mRNA (Fig. 3). Infection with the control adenovirus did not affect NCX mRNA. These results suggest that Rho family protein other than RhoA is involved in the regulation of

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NCX1 mRNA.

Effect of LPC on NCX1-mRNA level

We previously showed that in vascular endothelial cells (Yokoyama et al., 2002) and guinea-pig cardiac ventricular cells (Li et al., 2002) LPC stimulates nonselective cation currents via activation of Rho-signaling pathway. Therefore we examined whether LPC-induced Rho activation causes accumulation of NCX1 mRNA. Treatment of H9c2 cells with 5 μM LPC for 24 hours significantly increased NCX1 mRNA. This effect of LPC was inhibited by expression of C3 exoenzyme (Fig 4). The LPC-induced increase in NCX1 mRNA was blocked by Flv (data not shown), which is consistent with our previous data obtained with cardiac ventricular myocytes and endothelial cells. These results support the view that NCX1 mRNA levels are regulated by a Rho signaling pathway.

Western blotting was used to see whether the increase in NCX1 mRNA caused by LPC was reflected in the NCX1 protein level. Anti-NCX1 antibody recognized a single band having the expected molecular mass of 120 kDa in extracts of H9c2 cells (Fig 5A). Incubating cells with 5 μM Flv and 5 μM LPC for 24 hours resulted in a decrease to 63% and in an increase to 123% of

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control NCX1 protein, respectively (Fig 5B), which are consistent with the RT-PCR results shown in Fig.4.

Detection of RhoB protein in H9c2 cells

Since the Flv-induced down-regulation of NCX1 mRNA was cancelled not only by GGPP but also by FPP, the signaling protein responsible for regulation of NCX1 mRNA levels might be isoprenylated either by GGPP or FPP. Among several C3 exoenzyme substrates, RhoB is known to receive such modulation (Adamson et al., 1992a). Western blot analysis with RhoB specific antibody revealed that H9c2 cells possess RhoB protein (Fig 6A). Incubating cells with 5 μM Flv for 24 hours resulted in a decrease to 44% of the control in membrane associated RhoB protein level and in an increase by 432 % in cytosolic RhoB protein level (Fig 6B), suggesting that Flv blocks isoprenylation of RhoB and its function (Stamatakis et al. 2002). Conversely, incubating cells with 5 μM LPC for 24 hours resulted in an increase to 157% of the control in membrane associated RhoB protein and in a decrease to 76% of the control in cytosolic RhoB protein (Fig 6B). These results suggest that RhoB could be a candidate regulator of NCX1 mRNA.

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Effect of RhoB siRNA on NCX1 mRNA and protein in H9c2 cells

To directly examine the role of RhoB in NCX1 mRNA expression, we used RhoB specific siRNA to inhibit endogenus RhoB function and compared with the effect of RhoA specific siRNA in H9c2 cells. In preliminary experiments, we tested several different transfection methods to load siRNAs in H9c2 cells, and highest transfection efficacy, which was still less than 30 %, was obtained with Polyfect transfection reagent. Even with such relatively low transfection efficacy, RT-PCR analyses revealed that siRNA for RhoA and RhoB selectively decreased their own target mRNA (Fig7A). Negative control siRNA had no effect on gene expressions of RhoA, RhoB or GPDH. Transfection with RhoB siRNA for 24 hours decreased NCX1 mRNA significantly to 71.3±0.02 % of the control cells (n= 6, Fig 7B). In contrast, RhoA siRNA did not have a significant effect on NCX1 mRNA. Western blot analysis revealed that siRNA for RhoB selectively decreased its own target protein (Fig8A). Transfection with RhoB siRNA for 72 hours decreased NCX1 protein significantly to 50.2±0.05 % of the control cells (n= 3, Fig 8B). In contrast, negative control and RhoA siRNAs did not have a significant effect on RhoB and NCX1 protein. These results suggest that RhoB, but not RhoA, is involved in the regulation of NCX1 mRNA and protein.

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Effect of Flv on NCX1 mRNA stability

To investigate whether Flv affects NCX1 mRNA stability, cells were incubated with the transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB) at 50 μg/ml. Figure 9 shows that in the presence of DRB both NCX1 and GPDH mRNA levels slowly decreased and the rate of decay was faster for NCX1 than that for GPDH. Under these conditions, Flv accelerated the rate of decrease in NCX1-mRNA without affecting that of GPDH-mRNA. Assuming that NCX1 gene transcription was completely inhibited by DRB, half-life of NCX1 mRNA in control cells was about 18 hours, which was shortened by Flv to about 10 hours. An opposite effect (increase in NCX1 mRNA half-life) was observed in LPC-treated cells (Data not shown).

Effect of Flv on rat cardiac NCX1 mRNA level *in vivo*.

To examine whether Flv affects the cardiac NCX1 mRNA levels i*n vivo*, rats were fed for one week with either control or Flv (1 mg/g)-containing diet. Before the experiment, the body weight did not differ between control and Flv groups. After the one week, the body weight of control group increased from 116±2.7 g to 128±2.3 g (n=5). In contrast, the body weight

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of Flv group did not increase at all (from 112±1.6 g to 111±2.2 g, n=5). The total amount of diet taken by Flv group was slightly less than that in control group (about 90 % of control) and averaged daily dose of Flv was about 60 mg/kg/day. As shown in Figure 10, cardiac NCX1 mRNA levels in Flv-fed rats were significantly deceased to 65 % of that in control rats.

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Discussion

The present study demonstrated that Flv decreases NCX1 mRNA in H9c2 cells accompanied by a parallel decrease in NCX1 protein. This effect of Flv on the level of NCX1 mRNA can be explained by the inhibition of HMG-CoA reductase, because it was prevented by Mev. It is generally assumed that the effects of statins result from competitive inhibition of cholesterol synthesis (Goldstein and Brown, 1990; Levine et al., 1995). However, several recent studies demonstrated that, in addition to lowering cholesterol, statins prevent the synthesis of various isoprenoids, such as FPP and GGPP (Grunler, et al. 1994). These isoprenoids are important lipid attachments for post-translational modification of a variety of small GTP-binding proteins, such as Ras and Rho small G-proteins (Casey, 1995). Flv down-regulation of NCX1 mRNA was prevented by GGPP and FPP, suggesting that small G-

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proteins are targets for the effect of Flv. This was supported by the observation that the down-regulation of NCX1 mRNA by Flv was mimicked by the expression of C3 toxin, a specific inhibitor of Rho family G-proteins. These results indicate that the level of NCX1 mRNA is regulated by the Rho signaling pathway in H9c2 cells.

Geranylgeranylation is required for membrane association of activated Rho (Casey, 1995). Many studies demonstrated that unlike GGPP, FPP usually does not reverse statin inhibition of Rho-mediated responses (Laufs and Liao, 1998; Wassmann et al, 2001; Yokoyama et al, 2002). However, in this study, both GGPP and FPP prevented the down-regulation of NCX1 mRNA by Flv. FPP is required for isoprenylation of Ras. However, Ras-mediated signaling seems to have a minor role, if any, in the regulation of NCX1 mRNA levels, because expression of constitutive active mutant of Ras did not modulate NCX1 mRNA levels. In addition, we could not observe significant alteration of NCX1 mRNA by expression of constitutive active mutant of RhoA. These results together with the effects of C3 exoenzyme suggest that Rho family proteins other than RhoA may be responsible for regulation of NCX1 mRNA levels.

Involvement of certain Rho family protein in the regulation of NCX1

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mRNA levels was also suggested by experiments with LPC. We previously showed that in vascular endothelial cells (Yokoyama et al., 2002) and guinea-pig cardiac ventricular cells (Li et al., 2002) LPC induces nonselective cation currents via activation of Rho-signaling pathway. In the present study, we found that LPC up-regulated the NCX1 mRNA in H9c2 cells. This effect of LPC was inhibited by expression of C3 exoenzyme, suggesting that an activation of Rho family G-protein is involved in LPC-induced increase in NCX1 mRNA.

Among several C3 exoenzyme substrates, RhoB is known to be isoprenylated not only by GGPP but also by FPP (Adamson et al., 1992). We identified RhoB protein in H9c2 cells (Fig6). In addition, membrane associated RhoB was decreased by Flv treatment, but increased by LPC, reflecting that RhoB function is inhibited by depletion of isoprenoids by Flv, but stimulated by LPC in H9c2 cells. These results may suggest that RhoB is a regulator of NCX1 mRNA level. However, recent studies demonstrated that differential isoprenoid modification of RhoB altered its function and cellular localization. Thus, farnesylated RhoB localizes to the plasma membrane (Lebowitz et al., 1995) and induces growth promotion (Lebowitz et al., 1997), whereas geranylgeranylated RhoB localizes to multivesicular late

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endosomes (Lebowitz et al., 1995) and has apoptotic and growth-inhibitory effects (Du and Prendergast, 1999). Nevertheless, possible involvement of RhoB in the regulation of NCX1 mRNA was further supported by experiments with RhoB siRNA. We found that NCX1 mRNA and protein in H9c2 cells were decreased by transfection of siRNA specific for RhoB but not for RhoA. When H9c2 cells were treated with Flv, we observed not only a decrease in membrane associated RhoB, but also a marked increase in cytosolic RhoB. This is consistent with the interpretation that isoprenylation is necessary for rapid turnover of RhoB (Stamatakis et al, 2002). The cytosolic RhoB accumulated by Flv in H9c2 cells must be non-prenylated inactive form, which can not be utilized for activation by GTP loading. Therefore, the regulation of NCX1 mRNA levels may be a down stream event of activated RhoB. Although the molecular mechanisms remain to be elucidated, our data suggest that RhoB plays an important role in the regulation of NCX1 mRNA levels.

On the basis of the statin effects, Rho-mediated alterations of mRNA levels have been demonstrated with various proteins. For example, statins up-regulate the mRNA expressions of endothelial nitric oxide synthase (Laufs and Liao, 1998) and tissue plasminogen activator (Essig et al. 1998),

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but down-regulate the expression of plasminogen activator inhibitor-1 (Ishibashi et al, 2002) and AT1 angiotensin II receptor (Wassmann et al, 2001). The present results show for the first time that NCX1 mRNA levels are regulated by Rho-family G-protein RhoB. Furthermore, the results obtained with DRB-treated cells suggest that the down regulation of NCX1 mRNA by Flv is due to a decrease in NCX1 mRNA stability, although a direct effect of Flv on NCX1 transcription remains to be investigated. Increased mRNA stability by the Rho signaling pathway was demonstrated with the angiotensin AT1 receptor (Wassmann et al, 2001). In addition, an opposite effect of Rho (decrease in mRNA stability) was described with endothelial nitric oxide synthase (Laufs and Liao, 1998), suggesting that the Rho signaling pathway may be involved in the regulation of mRNA stability. Further studies are necessary to clarify how RhoB signaling pathway regulates NCX1 mRNA stability.

A regulatory role of the Rho signaling pathway in NCX1 expression provides a new perspective for understanding the mechanism underlying pathophysiological changes in cardiac NCX1 expression. In many animal models of cardiac hypertrophy and heart failure, both NCX expression and activity are increased (Hatem et al., 1994; Studer et al., 1994; Hasenfuss et

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al., 1999; Pogwizd et al., 1999; Gomez et al., 2002). The myocardial Rho signaling pathway is activated by various extracellular signaling molecules, such as angiotensin II (Aoki et al, 1998) and endothelin (Kuwahara et al., 1999), which are also considered to be factors mediating cardiac hypertrophy and heart failure. In this study, we found that chronic treatment of rat with Flv *in vivo* significantly decreased cardiac NCX1 mRNA. Although the dose of Flv used in this study is higher than that of clinical medication, this result indicate that the effects of Flv on NCX1 mRNA observed in H9c2 cells occur in the cardiac tissue *in vivo.*

In conclusion, we have found that inhibition of HMG-CoA reductase in H9c2 cells leads to a decrease in NCX1 mRNA stability through an inhibition of the RhoB signaling pathway. Our data also indicate that activation of Rho increases NCX1 expression. These observations show for the first time that Mev-derived metabolites play regulatory roles in the expression of NCX1. These results may have important implications for understanding the mechanism of altered NCX1 expression in cardiac hypertrophy and heart failure.

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Footnote:

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Figure Legends

Fig.1. Effects of Flv on NCX1 mRNA levels in H9c2 cells. A, H9c2 cells were treated with 5 μM Flv for indicated time periods, and NCX1 mRNA levels were examined by RT-PCR. GPDH mRNA levels were also determined as internal control. B, Time-dependent effects of Flv on NCX1 mRNA levels. Data were corrected with GPDH levels and expressed as fold of untreated control cells. C, Concentration-dependent effects of Flv on NCX1 mRNA levels. H9c2 cells were treated with different concentrations of Flv for 24 hours. Data shown are the mean ± S.E.M. $*p < 0.05$ $**p < 0.01$ compared with the control by Dunnett's test.

Fig.2. Effect of Mev, GGPP and FPP on Flv-induced down-regulation of NCX1 mRNA expression. H9c2 cells were treated with Flv for 24 h in the presence or absence of 100 μM Mev, 2.5 μM GGPP or 2.5 μM FPP. NCX mRNA expression was determined by RT-PCR. Data were corrected with GPDH levels and expressed as fold of untreated control cells. $**p < 0.01$ compared with the control by Dunnett's test.

Fig.3. Effect of expression of C3 exoenzyme, constitutively active mutant of

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RhoA (RhoAV17) or Ras (RasV14) on NCX1 mRNA expression. H9c2 cells were infected with adenovirus vector or adenovirus encoding C3 exoenzyme, RhoAV17 and RasV14. NCX mRNA expression was determined by RT-PCR. Data were corrected with GPDH levels and expressed as fold of untreated control cells. $**p < 0.01$ compared with the control by Dunnett's test.

Fig.4. Effect of LPC on NCX1 mRNA expression. H9c2 cells were infected with control adenovirus vector or adenovirus encoding C3 exoenzyme. After 24 hours, cells were treated with or without 5 μM LPC for additional 24 hours. mRNA expression was determined by RT-PCR. NCX mRNA expression was determined by RT-PCR. Data were corrected with GPDH levels and expressed as fold of untreated control cells. $**p < 0.01$ compared with the control by Dunnett's test.

Fig.5. Effect of 5 μM Flv and LPC for 24 hours on NCX1 protein in H9c2 cells. A, Representative Western blot. B, Relative densities of each signal were expressed as fold of control cells. $**p < 0.01$ compared with the control by Dunnett's test.

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Fig.6. Detection of RhoB in H9c2 cells and modulation of its cellular localization by Flv and LPC. H9c2 cells were incubated for 24 hours in the presence or absence of 5 μM Flv or 5 μM LPC. Cells were then harvested with lysis buffer. Membrane and cytosol fractions were prepared and subjected to Western blot analysis using RhoB specific antibody. Similar results were obtained in three different experiments. A, Representative Western blot. B, Relative densities of each signal was expressed as fold of control cells. $**p < 0.01$ compared with the control by Dunnett's test.

Fig.7. Effect of siRNA against RhoA or RhoB on NCX1 mRNA expression. A, H9c2 cells were transfected with siRNA against RhoA, RhoB and negative control. mRNA levels of RhoA, RhoB, NCX1 and GPDH were examined by RT-PCR. B, Relative densities of NCX1 signals were expressed as folds of that of untransfected control cells. Data were corrected with corresponding GPDH. Data shown are the mean ± S.E.M. $**p < 0.01$ compared with the control by Dunnett's test.

Fig.8. Effect of siRNA targeting RhoB on NCX1 protein. A, H9c2 cells were transfected with siRNA targeting RhoA, RhoB or negative control. Protein

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levels of RhoB and NCX1 were examined by Western blot. B, Relative densities of NCX1 protein bands were expressed as folds of that of untransfected control cells. Data shown are the mean ± S.E.M. $^{**}p < 0.01$ compared with the control by Dunnett's test.

Fig.9. Effect of Flv on NCX1 mRNA stability in the presence of DRB, a transcription inhibitor. A. RT-PCR products from H9c2 cells treated with or without 5 μM Flv in the presence of 50 μg/ml DRB. Total RNA was isolated at the indicated time points. B, C Time-dependent changes of GPDH and NCX1 mRNA levels. Data were expressed as fold of untreated cells (0 hours). $^{**}p < 0.01$ compared with the control by Dunnett's test.

Fig.10. Effect of one week treatment of rats with Flv *in vivo* on the cardiac NCX1 mRNA expression. Rats were fed with normal diet (control) or that containing 1 mg/g Flv for one week. A, Total RNA was isolated from heart, and RT-PCR was performed with NCX1 and GPDH primers. B, Real-time PCR was used to determine NCX mRNA levels. Data were calculated relative to internal control gene (GPDH) and are expressed as fold of contol. $^{**}p < 0.01$ versus control.

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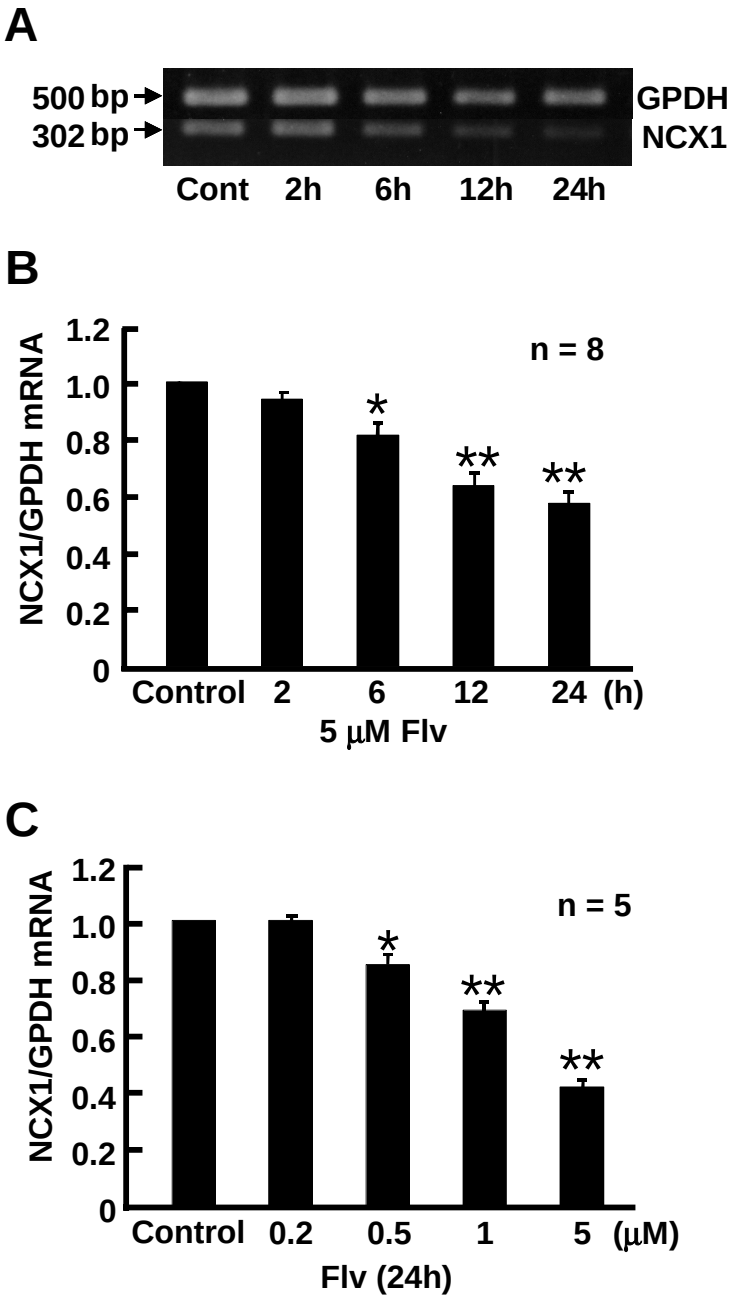

Fig. 1

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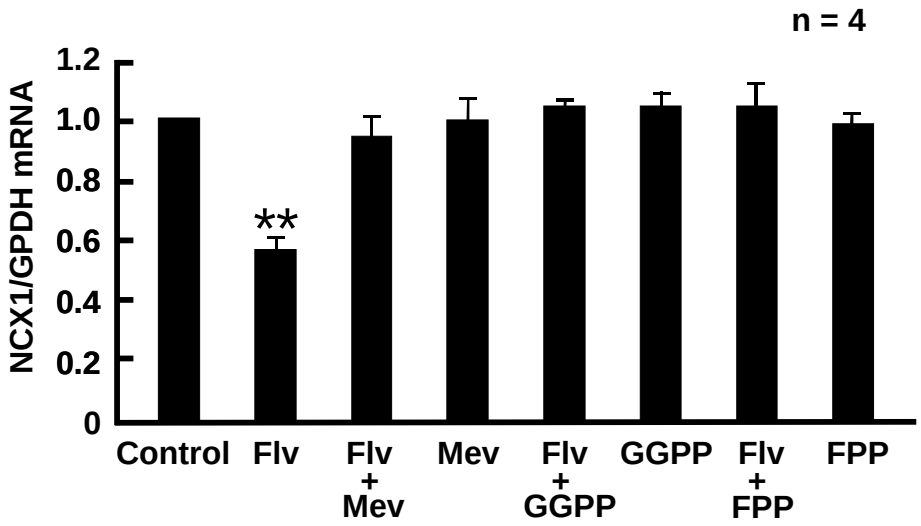

Fig. 2

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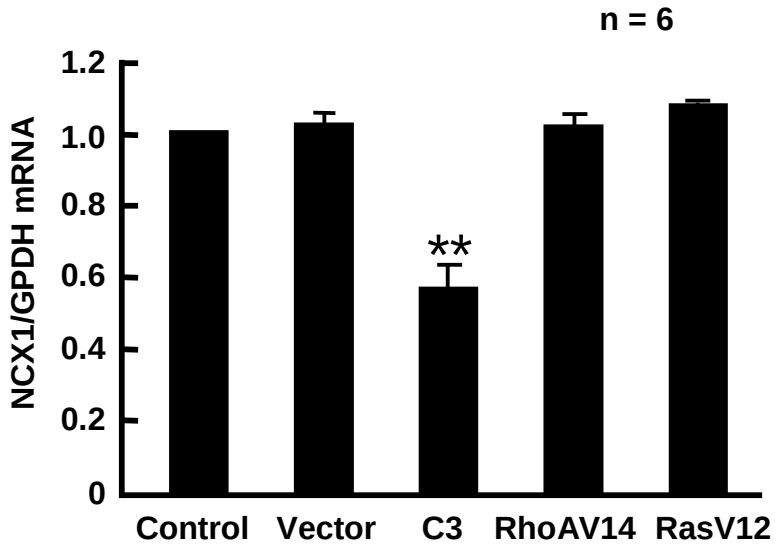

Fig. 3

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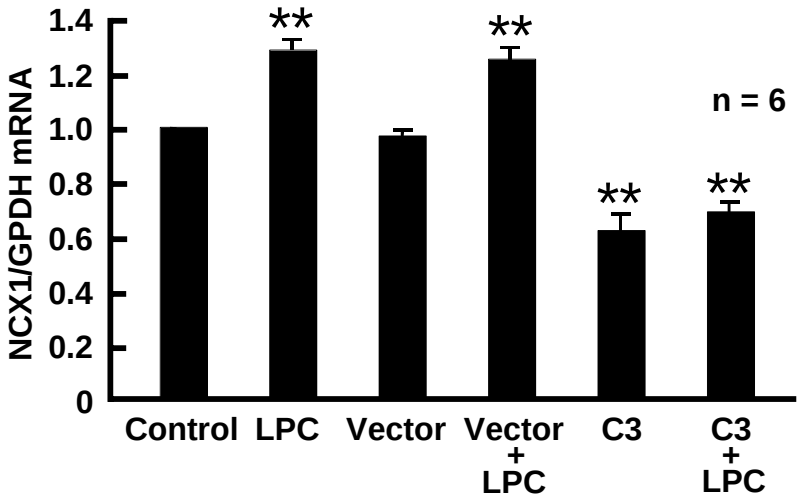

Fig. 4

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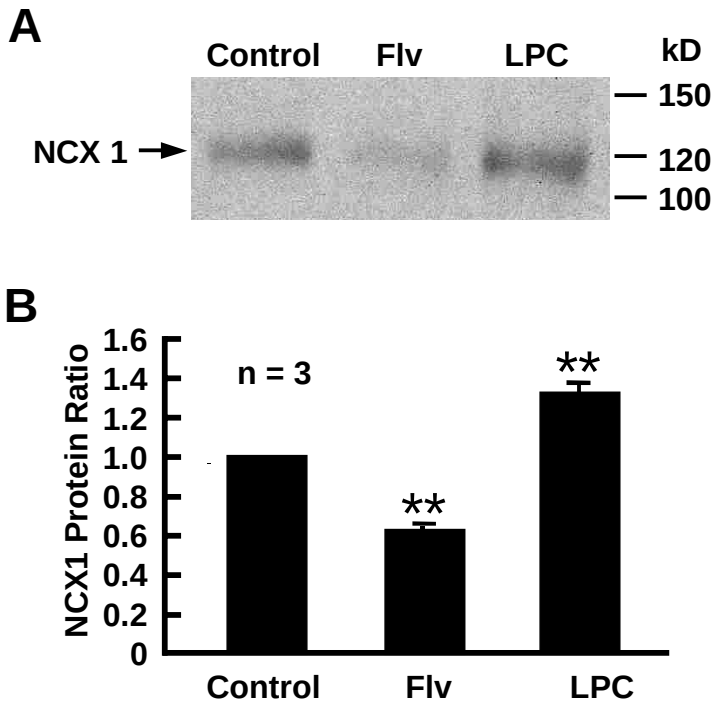

Fig. 5

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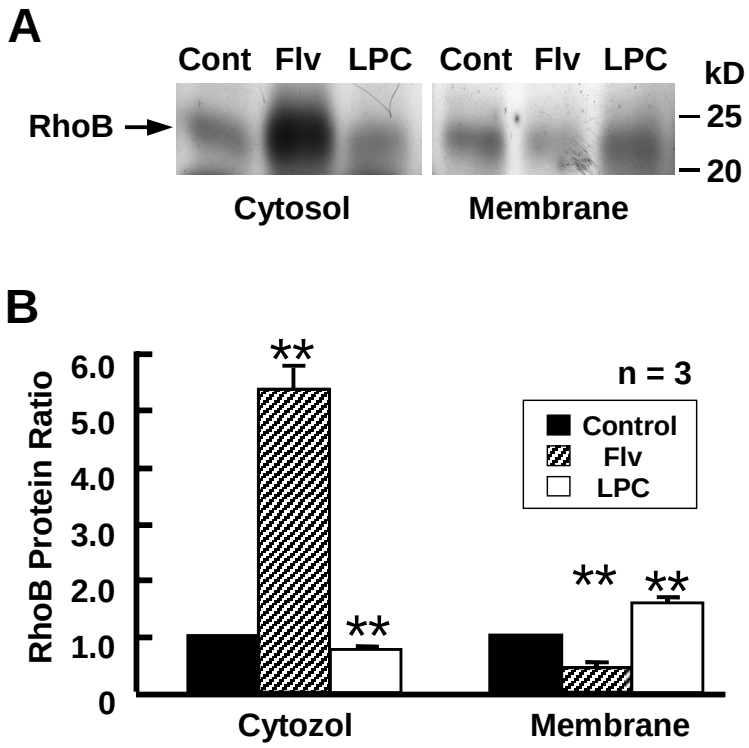

Fig. 6

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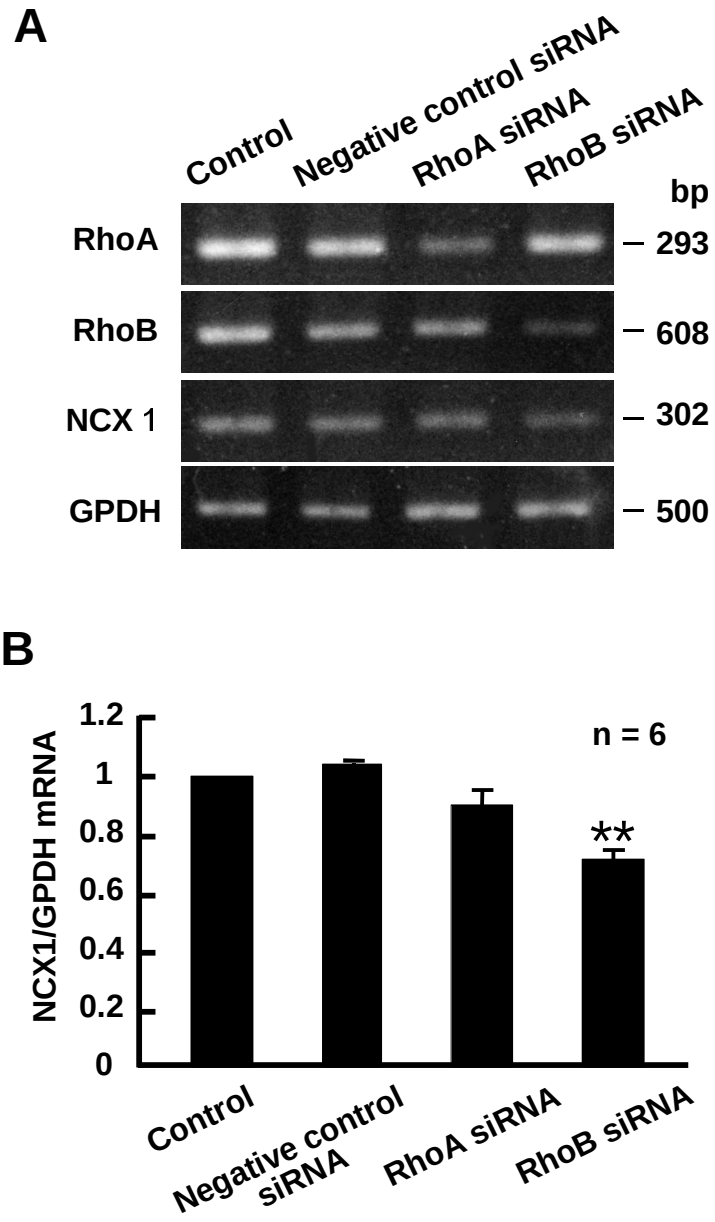

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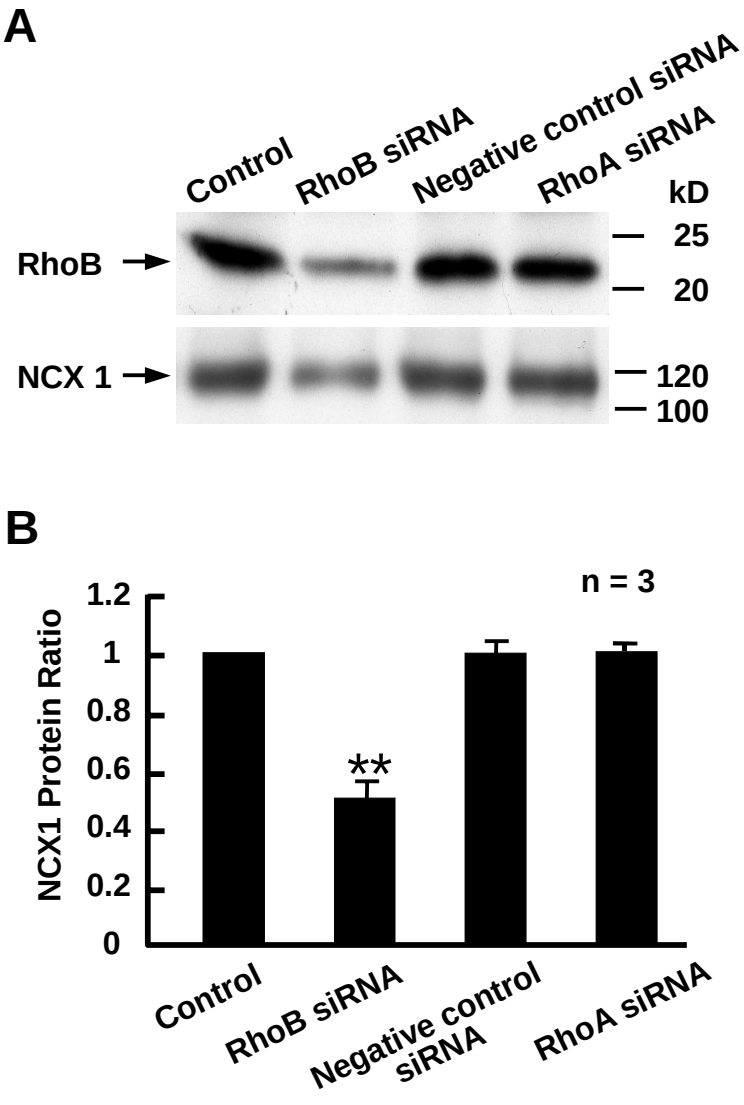

Fig. 8

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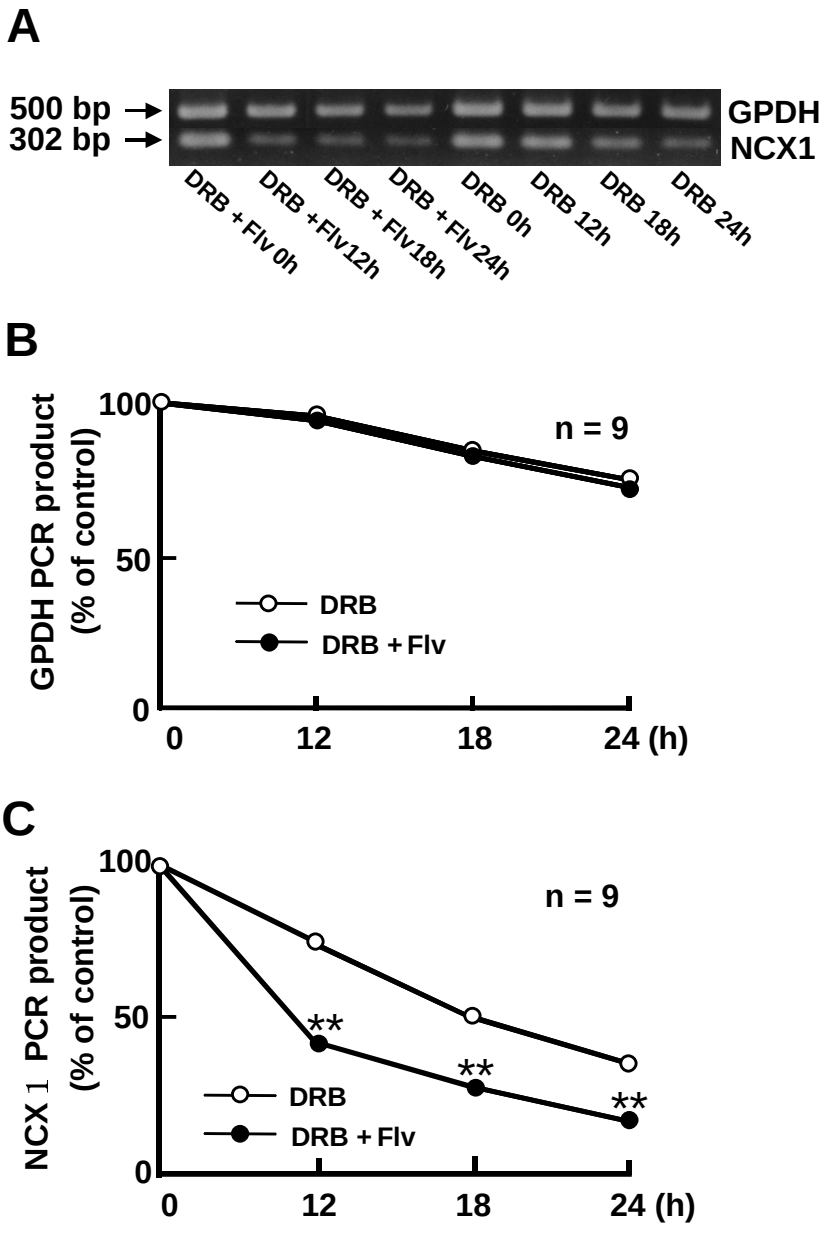

Fig. 9

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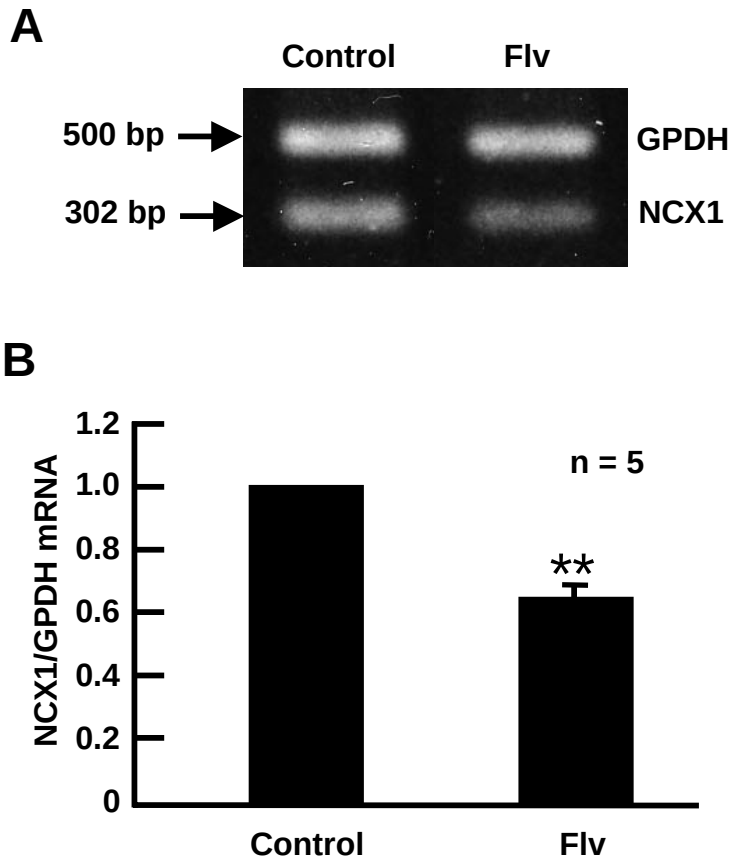

Fig. 10

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