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Opening of the Adenosine Triphosphate-Sensitive Potassium Channel Attenuates Cardiac Remodeling Induced by Long-Term Inhibition of Nitric Oxide Synthesis

Role of 70-kDa S6 Kinase and Extracellular Signal-Regulated Kinase

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OBJECTIVES We examined whether the adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel openers (KCOs) block myocardial hypertrophy and whether the 70-kDa S6 kinase (p70S6K) or extracellular signal-regulated kinase (ERK)-dependent pathway is involved.

BACKGROUND Long-term inhibition of nitric oxide (NO) synthesis induces cardiac hypertrophy independent of blood pressure, by increasing protein synthesis in vivo. The KCOs attenuate calcium overload and confer cardioprotection against ischemic stress, thereby preventing myocardial remodeling.

METHODS Twelve Wistar-Kyoto rat groups underwent eight weeks of the drug treatment in combination with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), the inactive isomer D^{ω}-nitro-L-arginine methyl ester, KCOs (nicorandil, 3 and 10 mg/kg per day, or JTV-506, 0.3 mg/kg per day), or the K_{ATP} channel blocker glibenclamide. The L-NAME was also used with hydralazine, the p70S6K inhibitor rapamycin, or the mitogen-activated protein kinase inhibitor PD98059. Finally, the left ventricular weight (LVW) to body weight (BW) ratio was quantified, followed by histologic examination and kinase assay.

RESULTS The L-NAME increased blood pressure and LVW/BW, as compared with the control agent. The KCOs and hydralazine equally cancelled the increase in blood pressure, whereas only KCOs blocked the increase in LVW/BW and myocardial hypertrophy induced by L-NAME. The L-NAME group showed both p70S6K and ERK activation in the myocardium (2.3-fold and 2.0-fold increases, respectively), as compared with the control group, which was not reversed by hydralazine. Selective inhibition of either p70S6K or ERK blocked myocardial hypertrophy. The KCOs prevented the increase in activity only of p70S6K. Glibenclamide reversed the effect of nicorandil in the presence of L-NAME.

CONCLUSIONS The KCOs modulate p70S6K, not ERK, to attenuate myocardial hypertrophy induced by long-term inhibition of NO synthesis in vivo. (J Am Coll Cardiol 2002;40:991–7) © 2002 by the American College of Cardiology Foundation

Recently, angiotensin II, the key product of the renin-angiotensin system, has been established as one of the critical factors for myocardial hypertrophy (1,2) and fibrosis (3), as well as other stimuli (4–6), such as norepinephrine, phenylephrine, endothelin-1, and peptide growth factors. These histologic changes are accepted to be the main features of cardiac "remodeling," as the result of many cardiovascular diseases (7,8). Recent studies have proposed that either extracellular signal-regulated kinase (ERK) (9–11) or 70-kDa S6 kinase (p70S6K) (9,12,13), which phosphorylate the 40S ribosomal protein S6 that regulates initiation of messenger ribonucleic acid translation (14,15), as well as 90-kDa ribosomal S6 kinase (6,11), is the key mediator of protein synthesis for hypertrophic changes induced by angiotensin II. Activation of p70S6K is reported to be involved in cardiac hypertrophy (13) and in the growth and proliferation of endothelial cells (16), smooth muscle cells (12), and fibroblasts (17).

In contrast, the adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels, located on the sarcolemma (18) and the inner membrane of the mitochondria (19) of cardiomyocytes, are involved in the cardioprotection against ischemia and reperfusion injuries (20,21). The opening of mitochondrial K_{ATP} channels is reported to alter the redox state of cardiomyocytes (22) and prevent mitochondrial Ca^{2+} overload (23), whereas the opening of sarcolemmal K_{ATP} channels modulates cardiac Na^{+}/K^{+}-ATPase activity (24), shortens the action potential duration (25), and prevents intracellular Ca^{2+} overload (26). Because Ca^{2+} activates protein kinase C (PKC) (27), which is reported to elicit cardiac hypertrophic changes through the activation of

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Abbreviations and Acronyms

ANOVA = analysis of variance
ATP = adenosine triphosphate
BW = body weight
D-NAME = D^{ω}-nitro-L-arginine methyl ester
ERK = extracellular signal-regulated kinase
K_{ATP} = adenosine triphosphate-sensitive potassium
KCO = adenosine triphosphate-sensitive potassium channel opener
L-NAME = N^{ω}-nitro-L-arginine methyl ester
LVW = left ventricular weight
NO = nitric oxide
p70S6K = 70-kDa S6 kinase
PKC = protein kinase C

ERK (28) or calmodulin-dependent protein kinases (29), the K_{ATP} channel openers (KCOs) could potently inhibit the hypertrophic changes induced by angiotensin II, possibly by preventing Ca^{2+}overload. However, there has been no study to test the effects of KCOs on cardiac hypertrophy.

To test this idea, we used the in vivo Wistar-Kyoto rat model with long-term inhibition of nitric oxide (NO) synthesis by N^{ω}-nitro-L-arginine methyl ester (L-NAME). Nitric oxide is known to mediate vasodilation, inhibit platelet aggregation and prevent leukocyte adhesion to endothelial cells (30). However, the main features of this model on the cardiovascular system are reported to be myocardial remodeling (hypertrophy and fibrosis), coronary remodeling (medial thickening and perivascular fibrosis) and hypertension, mediated through the renin-angiotensin system (9,31,32). We evaluated: 1) the myocardial structural changes; 2) the tissue p70S6K and ERK activity; and 3) the effects of KCOs (nicorandil [33] and JTV-506 [34]) on each variable.

METHODS

All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals (National Institutes of Health* [NIH] publication no. 85-23, revised 1985).

The S6 peptide (RRRLSSLRA, no. 12-124) was obtained from Upstate Biotechnology (Charlottesville, Virginia). Protein G/protein A–coupled beads were from Oncogene Sciences (Cambridge, Massachusetts). Nicorandil was obtained from Chugai Pharmaceuticals (Tokyo, Japan). JTV-506 was obtained from Japan Tobacco (Tokyo, Japan). The other drugs were from Sigma (St. Louis, Missouri).

Instrumentation. Eighty eight-week-old male Wistar-Kyoto rats were randomly classified into 12 groups. The control group received no treatment. The L-NAME group received 1 g/l of L-NAME in drinking water. The D^{ω}-nitro-L-arginine methyl ester (D-NAME, the inactive isomer of L-NAME) group received 1 g/l of D-NAME in drinking water. The L-NAME + hydralazine group received both L-NAME and hydralazine (120 mg/l in drinking water).

The L-NAME + JTV group, L-NAME + Ncr3 group, L-NAME + Ncr10 group, L-NAME + Rap group, and L-NAME + PD group received JTV-506 (0.3 mg/kg per day), nicorandil (3 or 10 mg/kg per day), nicorandil (10 mg/kg per day), rapamycin (a potent p70S6K inhibitor; 0.5 mg/kg per day), and PD98059 (a potent ERK kinase inhibitor; 5 mg/kg per day), respectively (orally by ravage), in addition to L-NAME (1 g/l in drinking water). In the Glib group, L-NAME + Glib group, and L-NAME + Ncr10 + Glib group, the K_{ATP} channel blocker glibenclamide (5 mg/kg per day; orally by gavage) was given in combination with both L-NAME and nicorandil (10 mg/kg per day). We decided on the doses of hydralazine, JTV-506, nicorandil, rapamycin, PD98059, and glibenclamide, according to our preliminary experiments (data not shown)

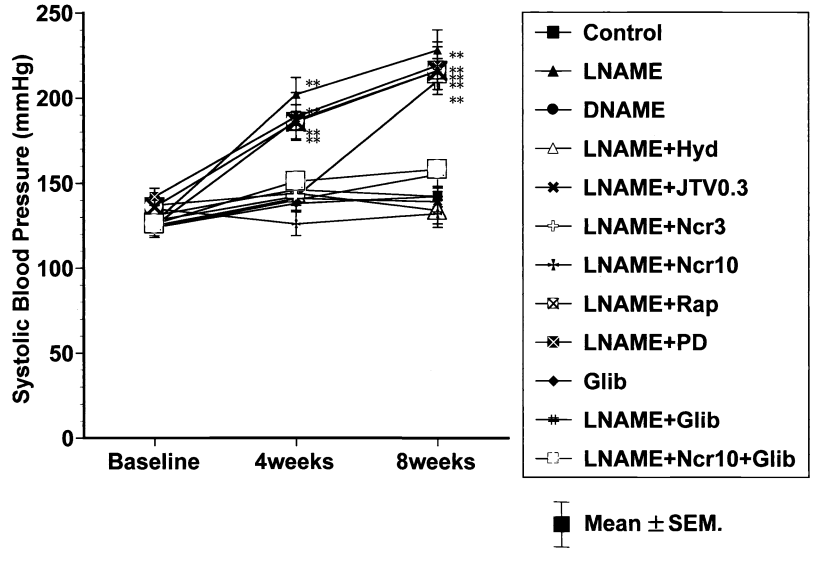

Figure 1. Time-course changes in systolic blood pressure. See text for details. Glib = glibenclamide; Hyd = hydralazine; JTV0.3 = JTV-506 at 0.3 mg/kg per day; Ncr3 and Ncr10= nicorandil at 3 and 10 mg/kg per day, respectively; PD = PD98059; Rap = rapamycin. $^{**}p < 0.01$ vs. control group.

Table 1. Changes in Body Weight and Left Ventricular Weight in Each Group

		Body Weight (g)		
	n	Baseline	4 Weeks	8 Weeks
Control	12	294 ± 4	352 ± 6*	392 ± 10*
L-NAME	12	292 ± 7	326 ± 9†‡	322 ± 13‡§
D-NAME	8	298 ± 3	365 ± 6*	399 ± 9*
L-NAME + Hyd	8	302 ± 4	350 ± 9*	379 ± 8*
L-NAME + JTV0.3	8	292 ± 3	345 ± 8*	348 ± 15*†
L-NAME + Ncr3	8	299 ± 6	349 ± 9*	372 ± 11*
L-NAME + Ncr10	8	294 ± 7	336 ± 8*	365 ± 7*
L-NAME + Rap	8	297 ± 8	329 ± 4‡	360 ± 10*
L-NAME + PD	6	288 ± 5	342 ± 5*	368 ± 7*
Glib	8	289 ± 4	344 ± 4*	380 ± 4*
L-NAME + Glib	8	290 ± 8	336 ± 9*	333 ± 6*†
L-NAME + Ncr10 + Glib	8	288 ± 6	328 ± 6†‡	338 ± 7*†

		LV Weight (mg)
	n	8 Weeks
Control	12	812 ± 16
L-NAME	12	939 ± 27†
D-NAME	8	819 ± 18
L-NAME + Hyd	8	1,079 ± 37§
L-NAME + JTV0.3	8	768 ± 14
L-NAME + Ncr3	8	959 ± 19†
L-NAME + Ncr10	8	826 ± 20
L-NAME + Rap	8	759 ± 22
L-NAME + PD	6	909 ± 44
Glib	8	784 ± 19
L-NAME + Glib	8	942 ± 35†
L-NAME + Ncr10 + Glib	8	909 ± 25

*$p < 0.01$ vs. baseline. †$p < 0.05$ vs. control group. ‡ $p < 0.05$ vs. baseline. §$p < 0.01$ vs. control group. Data are presented as the mean value ± SEM.

D-NAME and L-NAME = D^{ω}- and N^{ω}-nitro-L-arginine. methyl ester, respectively; Glib = glibenclamide; Hyd = hydralazine; JTV0.3 = JTV-506 at 0.3 mg/kg per day; Ncr3 and Ncr10 = nicorandil at 3 and 10 mg/kg per day, respectively; PD = PD98059; Rap = rapamycin.

and previous report (9). Rats from all groups were housed in a viral antigen-free facility and were fed with normal rat chow for eight weeks. Both systolic blood pressure and heart rate were measured by the tail-cuff method. After eight weeks of treatment, all rats were anesthetized with an intraperitoneal injection of thiopentobarbital and then sacrificed by exsanguination.

Histologic examination. Excised hearts were weighed, cut and carefully scanned, as described previously (9,35). The morphometry of left ventricular myocytes was assessed, and the cross-sectional area was measured in cardiomyocytes, according to a previous report (31).

Assay for p70S6K and ERK activity. The specific activity of p70S6K was determined by ^{32}P incorporation into the S6 peptide in the immune complex, as described (36–38). The specific activity of ERK was determined, as described previously (39). The experiments were performed twice for each sample.

Statistical analysis. Data are expressed as the mean value ± SEM. Paired data were compared by the Student *t* test. Comparisons of p70S6K activity, ERK activity, hemodynamic variables (e.g., systemic blood pressure, heart rate), body weight (BW), left ventricular weight (LVW) and cardiomyocyte cross-sectional area were performed by one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparisons *t* test. A comparison of time-course changes in systemic blood pressure was performed by two-way repeated measures ANOVA, followed by the multiple comparisons tests. A p value of <0.05 was considered statistically significant.

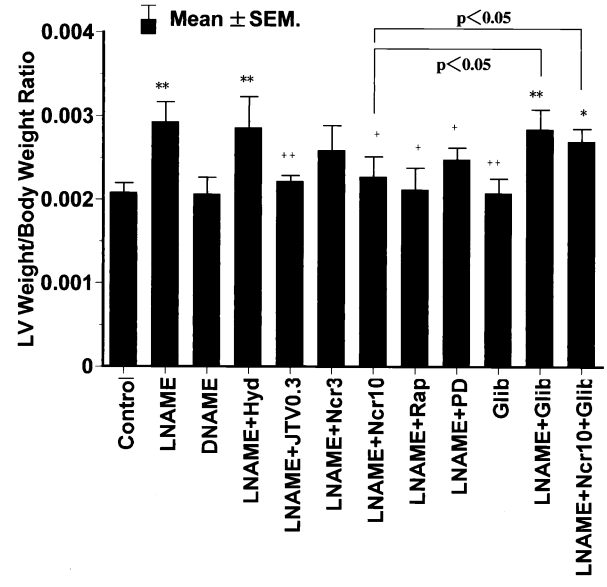

Figure 2. The left ventricular (LV) weight /body weight ratios. *$p < 0.05$ and **$p < 0.01$ vs. control group. †$p < 0.05$ and ††$p < 0.01$ vs. L-NAME group. Abbreviations as in Figure 1.

RESULTS

Arterial pressure, heart rate, and BW. Before treatment, systemic blood pressure was comparable among the 12 groups studied. After eight weeks of treatment, systemic blood pressure was comparable in each group, except in the L-NAME, L-NAME + Ncr3, L-NAME + Rap, L-NAME + PD, and L-NAME + Glib groups, which had higher levels ($p < 0.01$) than those in the control group (Fig. 1). The increase in blood pressure in this model was reversed by a dose as high as 10 mg/kg per day of nicorandil or hydralazine. The heart rate was comparable among the 12 groups studied and did not change significantly throughout this study. Body weight increased significantly in all groups, although the L-NAME, L-NAME + JTV, L-NAME + Glib, and L-NAME + Ncr10 + Glib groups had a lower BW than that of the control group (Table 1).

Myocardial hypertrophy and LVW/BW ratio. After eight weeks of treatment, the L-NAME, L-NAME + Hyd, L-NAME + Ncr3, and L-NAME + Glib groups had a significantly higher LVW than that of the control group (Table 1).

However, a significant increase ($p < 0.01$) in the LVW/BW ratio was observed in the L-NAME, L-NAME + Hyd, L-NAME + Glib, and L-NAME + Ncr10 + Glib

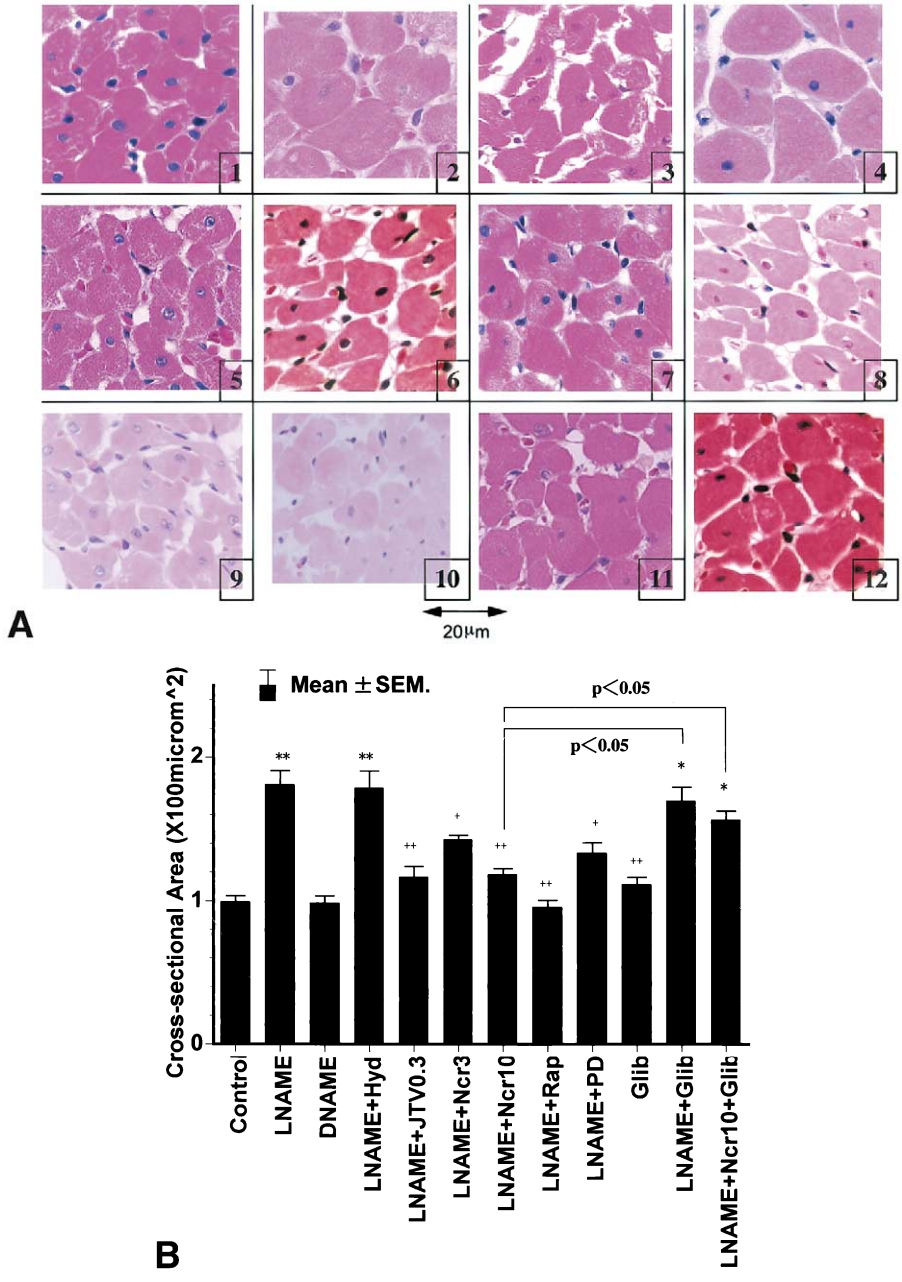

Figure 3. Representative histologic findings of myocytes **(A)** and myocyte cross-sectional areas **(B)**. *$p < 0.05$ and **$p < 0.01$ vs. control group. †$p < 0.05$, ††$p < 0.01$ vs. L-NAME group. 1 = control group; 2 = L-NAME treatment; 3 = D-NAME treatment; 4 = L-NAME + Hyd treatment; 5 = L-NAME + JTV treatment; 6 = L-NAME + Ncr3 treatment; 7 = L-NAME + Ncr10 treatment; 8 = L-NAME + Rap treatment; 9 = L-NAME + PD treatment; 10 = Glib treatment; 11 = L-NAME + Glib treatment; and 12 = L-NAME + Ncr10 + Glib treatment. Abbreviations as in Figure 1.

groups, as compared with the control group (Fig. 2). Either a higher dose of nicorandil, JTV-506, rapamycin or PD98059, but neither a lower dose of nicorandil nor hydralazine, prevented the increase in LVW/BW induced by L-NAME (Fig. 2). The LVW/BW ratio in the L-NAME + Ncr10 + Glib group was significantly higher than that in the L-NAME + Ncr10 group.

Representative histologic findings of the cross-sectional areas of cardiomyocytes are shown in Figure 3A. The cross-sectional areas of cardiomyocytes in the L-NAME group were significantly ($p < 0.01$) greater than those of the control group (Fig. 3B). Higher and lower doses of nicorandil, JTV-506, rapamycin or PD98059, but not hydralazine, prevented the increase in the cross-sectional areas of cardiomyocytes induced by L-NAME (Fig. 3B). As seen in LVW/BW, the cross-sectional area of cardiomyocytes in the L-NAME + Ncr10 + Glib group was significantly higher than that in the L-NAME + Ncr10 group.

Activity of p70S6K and ERK in cardiac tissue. The activity of p70S6K and ERK in cardiac tissue in the L-NAME group was higher ($p < 0.01$) than that in the

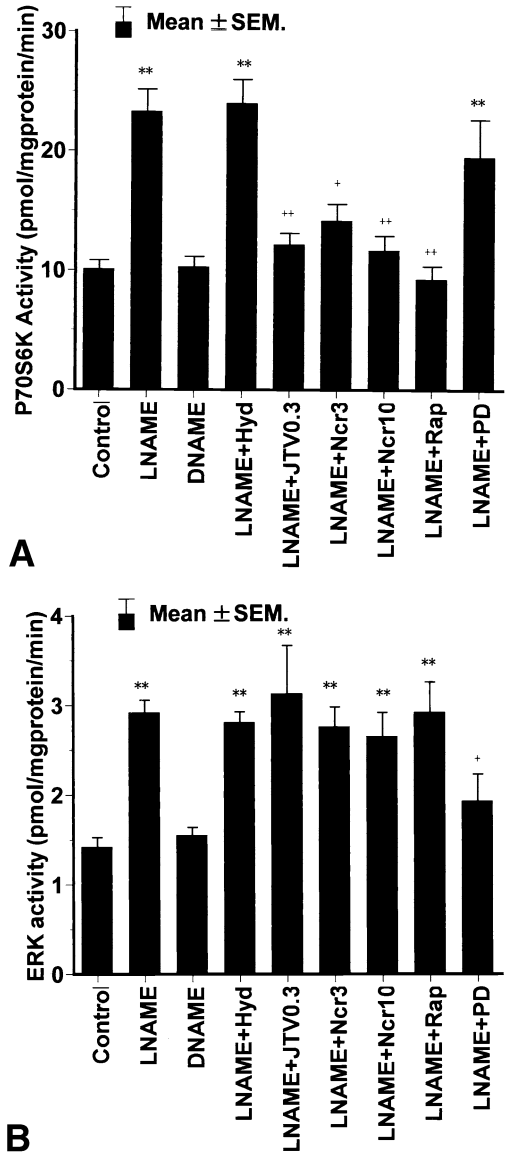

Figure 4. Activity of P70S6K **(A)** and ERK **(B)** in myocardial tissue. See text for details. $^{**}p < 0.01$ vs. control group. †$p < 0.05$ and ††$p < 0.01$ vs. L-NAME group. Abbreviations as in Figure 1.

control group. Nicorandil, JTV-506, or rapamycin, but not hydralazine nor PD98059, prevented the increase in p70S6K activity induced by L-NAME (Fig. 4A). Only PD98059 prevented the increase in ERK activity induced by L-NAME (Fig. 4B).

DISCUSSION

We have demonstrated that: 1) the myocardial hypertrophy induced by long-term inhibition of NO synthesis with L-NAME is reduced by KCOs in a dose-dependent manner, but not by hydralazine; 2) the K_{ATP} channel blocker glibenclamide abolished this hypertrophy-reducing effect of KCOs, whereas glibenclamide, per se, did not influence the untreated heart; and 3) p70S6K activity in the myocardium increases in rats treated with L-NAME, which was reduced by either KCOs or rapamycin, but not by either hydralazine or PD98059. Here we have shown for the first time, to the best of our knowledge, that KCOs potentially prevent myocardial structural changes induced by long-term inhibition of NO synthesis, through regulating p70S6K activity, which is apart from ERK in vivo.

Features of myocardial hypertrophy induced by inhibition of NO synthesis. Long-term treatment with L-NAME inhibits NO synthesis (40) and induces arterial hypertension, cardiac hypertrophy, and remodeling (31). However, these structural changes in the heart are independent of arterial hypertension, as revealed by the data using hydralazine. Furthermore, we have reported that reduced plasma NO levels are linearly correlated with the severity of hypertension in patients with essential hypertension (41), suggesting that this model may represent the cardiac hypertrophy associated with clinical essential hypertension in vivo. A variety of previous reports have proposed there are several possible mechanisms by which the inhibition of NO synthesis induces myocardial structural changes: 1) increases in plasma renin activity (42) and local angiotensin-converting enzyme activity (31) and upregulation of angiotensin II receptors (43), which increase the effect of angiotensin II; 2) expression of plasminogen activator inhibitor-1 (44) or the fetal skeletal alpha-actin isoform (42); and 3) synthesis of growth factors in the endothelium (45,46). We have previously demonstrated (47) that inhibition of NO synthesis leads to PKC activation through a cyclic guanosine monophosphate-independent mechanism, which is also reported to be involved in the pathway of angiotensin II-induced cardiac hypertrophy (11,28). However, other reports have revealed that NO modifies the vascular structure by directly modulating the growth of vascular smooth muscle cells (48). Taken together, it is likely that activation of the local or, in part, systemic renin-angiotensin system and the associated increased release of growth factors may contribute to cardiovascular hypertrophy in both a PKC-dependent and -independent fashion in this model.

Differential regulation of myocardial remodeling by p70S6K and ERK. In the present study, either rapamycin or PD98059 independently blocked or attenuated myocardial remodeling. Furthermore, the KCOs prevented the increase in activity only of p70S6K and attenuated cardiac hypertrophy in a dose-dependent manner, which was reversed by the potent K_{ATP} channel blocker glibenclamide. Taken together, it is suggested that opening of the K_{ATP} channel potentially prevents myocardial structural changes in the present in vivo model by regulating p70S6K, apart from ERK. Accordingly, we have reported that inhibition of NO synthesis in the same model induces cardiac remodeling, along with activation of both p70S6K and ERK (9), and modulation by angiotensin-converting enzyme inhibitors or angiotensin II type 1 receptor blockers can equally attenuate these changes by alternatively inhibiting p70S6K and ERK (9). Other reports mention that these two

signaling pathways are independently involved in cardiac hypertrophy (49). These data sufficiently support our present results. p70S6K is reported to be activated by the cytokines and hormonal and growth factors through phosphoinositide-3 kinase activation (50), and it phosphorylates S6. Furthermore, p70S6K is known to phosphorylate a transcriptional factor—the cyclic adenosine monophosphate response-element modulator (51). Thus, p70S6K might be important in transcriptional, as well as translational, regulation for protein synthesis.

Clinical implications. The KCOs have already been recognized as vasodilators (33,34) or potent agents for cardioprotection against ischemic injury (20,21). We have shown here for the first time, to the best of our knowledge, the potency of KCOs in attenuating myocardial structural changes, as well as the suggestive effecting point, p70S6K, as another target to prevent myocardial remodeling. Because it is hard to reverse cardiac remodeling completely by various kinds of medications in clinical conditions, this study proclaims the good clinical outcome of therapy with KCOs against cardiac remodeling, especially in those with clinical hypertension. Further studies are needed to adapt and establish these strategies safely and effectively in clinical cardiology.

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