KMUP-1 inhibits hypertension-induced left ventricular hypertrophy through regulation of nitric oxide synthases, ERK1/2, and calcineurin

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Received 11 October 2011; accepted 4 November 2011
Available online 25 July 2012

Abstract  Hypertension can induce left ventricular hypertrophy (LVH), and the nitric oxide (NO) pathway plays an important role in the pathogenesis of cardiac hypertrophy. This study aimed to examine whether KMUP-1, a novel xanthine-based derivative, could inhibit LVH in spontaneously hypertensive rats (SHRs) and to investigate potential mechanisms underlying its antihypertrophic effects. Two groups of animals with chronic or subacute LVH were treated. In the chronic LVH group, KMUP-1 (10 or 30 mg/kg/d orally) was administered for 28 days to both normotensive rats and SHRs. In the subacute LVH group, KMUP-1 (0.5 mg/kg/d intraperitoneally) or sildenafil (0.7 mg/kg/d intraperitoneally) was administered for 10 days with or without co-treatment with the nitric oxide synthase (NOS) inhibitor N-omega-nitro-L-arginine (L-NNA; 20 mg/L orally). After treatment, the effects of KMUP-1 or sildenafil on hypertension, cardiac hypertrophy, survival, expression of the NO/soluble guanylate cyclase (sGC)/protein kinase G (NO/sGC/PKG) pathway in the aorta and left ventricle, and calcineurin A/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in the left ventricle were examined. In the chronic LVH group, the SHRs developed hypertension with LVH over the 28 days. KMUP-1 attenuated the hypertension and LVH, increased survival rate, enhanced endothelial NOS/cyclic guanosine monophosphate/PKG (eNOS/cGMP/PKG) and decreased inducible NOS (iNOS) expression in the...
Introduction

Left ventricular hypertrophy (LVH), which is characterized by increased ventricular mass, develops in response to numerous forms of cardiac stress, such as pressure or volume overload [1]. Prolonged hypertension causes vascular endothelial dysfunction and LVH, with associated downregulation of endothelial nitric oxide synthase (eNOS) in the endothelium [2–5]. eNOS and inducible NOS (iNOS) are both important for the production of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), while phosphodiesterase (PDE)-5A is the major enzyme for cGMP hydrolysis. Enhanced cGMP production prevents the hypertrophic signaling and antagonizes cyclic adenosine monophosphate, resulting in an increase of protein kinase G (PKG) in the heart [6]. Indeed, sildenafil, a PDE-5 inhibitor, has been shown to have antihypertrophic activities by blocking the degradation of cGMP [7,8].

Emerging evidence suggests that eNOS is involved in ischemic protection and preservation of vascular contractility [9]. In fact, eNOS can regulate impaired endothelial NO bioactivity in LVH, and the NOS inhibitor N-omega-nitro-L-arginine (L-NNA) can reduce vascular relaxation via the NO/cGMP pathway, thus worsening LVH. In addition, it has been recently demonstrated that extracellular signal-regulated kinase 1/2 (ERK1/2) and calcineurin A are both important mediators signaling pathogenesis of cardiac hypertrophy [10,11]. Therefore, a therapeutic strategy involving not only the NO pathway, but also ERK 1/2 or calcineurin signaling, may be beneficial in the treatment of hypertensive LVH.

KMUP-1 (Fig. 1), a xanthine-based eNOS/cGMP-enhancer, has been reported to increase eNOS expression, inhibit iNOS expression, and inhibit pulmonary hypertension [12–14]. We have previously shown that KMUP-1 can attenuate isoprenaline-induced cardiac hypertrophy and inhibit right ventricular hypertrophy induced by pulmonary artery hypertension [14,15]. However, its effects on LVH induced by hypertension and/or L-NNA have not been examined. In addition, whether these effects are associated with modulation of NOS in both the left ventricle and the aorta is unknown. Thus, the current study addressed these questions in rats with subacute or chronic hypertensive LVH. The effects of KMUP-1 and sildenafil were also compared.

Materials and methods

Materials

KMUP-1, used in the salt form KMUP-1·HCl, was synthesized in our laboratory. Sildenafil citrate was kindly supplied by Cadila Healthcare Ltd. (Maninagar, India). L-NNA, a nonselective NOS inhibitor, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-eNOS antibody was obtained from BD Biotechnology (New York, NY, USA), anti-PKG antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-iNOS, soluble guanylate cyclase (sGC)α1, calcineurin A, and β-actin antibodies from Sigma, and anti-phospho ERK1/2 antibody from Cell Signaling Technology Inc. (Danvers, MA, USA). KMUP-1·HCl was dissolved in distilled water and sildenafil was dissolved in vehicle (distilled water containing 0.5% methyl cellulose) for the experiments.

Experimental animals

Eight-week-old male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats weighing 200–250 g were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed under constant temperature and controlled illumination (light on between 7:30 am and 19:30 pm) for 1 week before the experiment, i.e., beginning at week 9. Food and water were available ad libitum. All protocols were approved by the Animal Care and Use Committee of Kaohsiung Medical University. The animal experiments were divided into two groups: the chronic LVH group (28 days) and the subacute LVH group (10 days).

Chronic LVH group (28 days)

In the chronic LVH group, WKY and SHR rats were studied in a protocol of 28 days. WKY rats were divided into WKY-control (WKY-CTL) and WKY-KMUP-1 groups. The WKY-CTL rats received vehicle, while the WKY-KMUP-1 group
received KMUP-1 (10 mg/kg/d). SHRs were further divided into SHR-control (SHR-CTL) and SHR-KMUP-1 groups. The SHR-CTL group received vehicle, and the SHR-KMUP-1 group received KMUP-1 (10 or 30 mg/kg/d, given by gavage).

Subacute LVH group (10 days)

In the subacute LVH group, SHRs were randomly assigned to six groups and studied in a protocol lasting 10 days. The SHR-CTL group received intraperitoneal saline vehicle injection daily for 10 days. The SHR-KMUP-1 group received intraperitoneal KMUP-1: HCl (molecular weight 438.5 amu) in a vehicle-containing dose of 0.5 mg/kg/d, and the SHR-sildenafil group received intraperitoneal sildenafil citrate (molecular weight 666.7) in a vehicle-containing dose of 0.7 mg/kg/d; i.e. both drugs were administered at almost equal molecular weights. The SHR-L-NNA group received L-NNA in drinking water (20 mg/L). The SHR-L-NNA + KMUP-1 group received both L-NNA (20 mg/L) and KMUP-1 (0.5 mg/kg/d). The SHR-L-NNA + sildenafil group received both L-NNA in drinking water and sildenafil (0.7 mg/kg/d). SHRs in all groups were fed with normal rat chow.

Systolic artery pressure

The systolic artery pressure (SAP) and heart rate were recorded without anesthesia using the indirect tail cuff method with a rat tail manometer-tachometer (MK-2000 Storage Pressure Meter; Muromachi Kikai Co., Ltd, Tokyo, Japan). The rats in the chronic group were restrained in a Plexiglass holder at a temperature of 37 °C for 15–20 minutes to raise their body temperature. The increased temperature leads to dilatation of the caudal artery, which allowed us to detect pressure pulse easily. In all rats, at least three consecutive measurements were obtained, and the average was reported as the SAP. Changes in SAP were calculated by subtracting the SAP at the beginning from the average was reported as the SAP. Changes in SAP were allowed us to detect pressure pulse easily. In all rats, at least three consecutive measurements were obtained, and the average was reported as the SAP. Changes in SAP were calculated by subtracting the SAP at the beginning from the SAP at the beginning of the study. Changes of SAP in the subacute group are not shown.

Survival and heart weight indices

The number of survivors in each group was recorded daily until the end of the study. After the defined treatment period, the rats were sacrificed by intraperitoneal injection of 40 mg/kg pentobarbital sodium. The heart was perfused with saline, and the heart weight and body weight were recorded. The heart weight index was calculated by dividing the heart weight by the body weight [9].

Plasma nitrite/nitrate

As previously described, plasma nitrite/nitrate (NOx) was measured by the Griess reaction [15]. In brief, blood was sampled from the aorta and centrifuged at 370 g for 20 minutes at 4 °C. The supernatants were incubated with the Griess reagent at room temperature. Ten minutes later, the absorbance was measured at 540 nm using an automatic plate reader, and the NOx concentrations were expressed as micromoles and calculated using a standard curve for NOx.

Myocardial cGMP

Frozen myocardial tissue samples in liquid nitrogen were ground to a fine powder in a stainless steel mortar. Once the liquid nitrogen had evaporated, the frozen tissue was weighed and homogenized in 10 volumes of 0.1 M HCl to stop the action of PDEs. Centrifugation was at 600 g at room temperature, and the supernatant was collected for quantitative immunoassay of cGMP according to the manufacturer’s instructions (Enzo Life Sciences International, Inc. Plymouth Meeting, PA, USA).

Western blot analysis

eNOS, iNOS, sGCα, PKG, calcineurin A, phospho-ERK1/2, and total ERK1/2 proteins were measured by western blotting as described previously [12–14]. Briefly, the aorta and left ventricle, isolated from surviving rats, were homogenized separately in ice-cold lysis buffer. After sonication, the homogenate was centrifuged at 13,000 g for 60 minutes at 4 °C, and the supernatant was recovered as the total cellular protein. Total protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% acrylamide gels, transferred to a polyvinylidine difluoride-plus membrane, and then blocked with 5% nonfat dry milk in Tris-buffered saline. Membranes were subsequently incubated with a 1:1000 dilution of eNOS, iNOS, PKG, calcineurin A, and ERK1/2 antibodies. Proteins were detected with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Chemicon, Temecula, CA, USA). The immunoreactive bands were detected by chemiluminescence reagents developed by Hyperfilm (Kodak, Rochester, NY, USA), and the loading control protein beta-actin was used in the analyses.

Statistical analysis

The results are expressed as mean ± standard error. Statistical differences were determined by Student t test in unpaired samples. Whenever a control group was compared with KMUP-1 and other treated groups, one-way analysis of variance (ANOVA) or two-way repeated-measures ANOVA was used. When ANOVA manifested a statistical difference, Dunnett’s test was applied. A value of p < 0.05 was considered significant.

Results

Effects on SAP and cardiac hypertrophy in the chronic LVH group

In the chronic LVH group, the control basal levels of SAP in WKY rats and SHRs were 124.4 ± 6.2 and 151 ± 3.9 mmHg, respectively. After 28 days, this increased to 134.6 ± 3.2 and 181.6 ± 5.7 mmHg, respectively. WKY rats treated with KMUP-1 (10 mg/kg/d orally) for 28 days did not show a significant reduction in the change in SAP (Fig. 2A). In SHRs, KMUP-1 (10 and 30 mg/kg/d orally) prevented the development of hypertension (Fig. 2A). As shown in Fig. 2B, SHRs developed cardiac hypertrophy, indicated by an
increased heart weight index compared with WKY rats. KMUP-1 (10 and 30 mg/kg/d) significantly attenuated the hypertrophic responses in SHRs.

Effects on survival and cardiac hypertrophy in the subacute LVH group

Kaplan–Meier survival plots of SHRs in the subacute LVH group are shown in Fig. 3A. At the termination of the study, the survival rates of the control SHRs and the KMUP-1-treated and sildenafil-treated groups were all 100%. The survival rate of the SHR-L-NNA group was 80% (12/15). The SHR-L-NNA + KMUP-1 group and the SHR-L-NNA + sildenafil group both had an 87% survival rate (13/15), indicating that KMUP-1 and sildenafil tended to improve the survival of SHRs, although this did not reach a significant difference.

As shown in Fig. 3B, intraperitoneal injection of KMUP-1 (0.5 mg/kg/d) and sildenafil (0.7 mg/kg/d) for 10 days attenuated cardiac hypertrophy in SHRs. The L-NNA (20 mg/L) group had more profound cardiac hypertrophy compared with the SHR-CTL group. In the presence of L-NNA, neither KMUP-1 nor sildenafil caused a significant reduction in cardiac hypertrophy.
Effects on arterial eNOS, sGC, PKG, and iNOS

In the chronic LVH group, KMUP-1 (10 mg/kg/d orally) significantly increased the expression of eNOS, sGC, and PKG in the aorta of WKY rats (Fig. 4A, C, D). KMUP-1 (30 mg/kg/d orally) also increased the expression of eNOS, sGC, and PKG in SHRs. Expression of iNOS was profoundly increased in SHRs compared with the WKY-CTL group (Fig. 4B). Treatment with KMUP-1 (30 mg/kg/d orally) significantly attenuated the expression of iNOS in SHRs compared with the SHR-CTL group (Fig. 4B).

Effects on left ventricular eNOS and iNOS

In the chronic LVH group, oral administration of KMUP-1 (10 mg/kg/d) significantly increased ventricular eNOS expression in SHRs and WKY rats. The eNOS expression in the SHR-CTL and SHR-KMUP-1 groups was less than that in the WKY-CTL group (Fig. 5A). In the subacute LVH group, both KMUP-1 (0.5 mg/kg/d intraperitoneally) and sildenafil (0.7 mg/kg/d intraperitoneally) increased expression of eNOS in SHRs (Fig. 5B).

In the chronic LVH group, the increased expression of iNOS in SHRs was significantly decreased by treatment with KMUP-1 (10 mg/kg/d) (Fig. 5C). KMUP-1 (10 mg/kg/d) had no effect on iNOS in the WKY group. In the subacute LVH group, treatment with KMUP-1 (0.5 mg/kg/d intraperitoneally) or sildenafil (0.7 mg/kg/d intraperitoneally) significantly decreased the expression of iNOS in SHRs (Fig. 5D).

Effects on plasma NOx

In the chronic LVH group, we sampled the plasma of rats that received oral KMUP-1 (10 mg/kg/d) to measure NOx. As seen in Fig. 6A, there were no differences in plasma NOx

![Figure 4.](image-url)
levels in the WKY, WKY-KMUP-1, and SHR-CTL groups, whereas SHRs treated with KMUP-1 were found to have higher plasma NOx levels than the SHR-CTL group and the WKY-CTL group.

In the subacute LVH group, treatment with KMUP-1 and sildenafil significantly increased the plasma NOx in SHRs (Fig. 6B), whereas treatment with L-NNA significantly reduced the basal production of NOx (Fig. 6B). Treatment with KMUP-1 and sildenafil prevented this L-NNA-induced reduction (Fig. 6B).

Effects on left ventricular cGMP and PKG

In the chronic LVH group, ventricular cGMP and PKG levels in SHRs were lower than those in WKYs (Figs. 6C and 7A). KMUP-1 (10 mg/kg/d) significantly increased cGMP and PKG expression in the left ventricle of normotensive and hypertensive rats.

Similarly, in the subacute LVH group, as shown in Figs. 6D and 7B, both KMUP-1 (0.5 mg/kg/d intraperitoneally) and sildenafil (0.7 mg/kg/d intraperitoneally) significantly increased cGMP and PKG expression and abolished L-NNA-induced downregulation of cGMP and PKG. L-NNA alone significantly decreased cGMP and PKG expression in SHRs.

Effects on left ventricular calcineurin A and ERK1/2

In the chronic LVH group, (Fig. 8A, C), the SHR-CTL group had higher expression levels of calcineurin A and phosphorylated ERK1/2 than WKY-CTL rats ($p < 0.01$). Treatment with KMUP-1 (10 mg/kg/d orally) significantly attenuated the expression of calcineurin A and phosphorylated ERK1/2 in SHRs.

In the subacute LVH group, as seen in Fig. 8B and 8D, both KMUP-1 and sildenafil significantly inhibited the
expression of calcineurin A and phosphorylated ERK1/2. SHRs treated with L-NNA were found to have an increase in calcineurin A and phosphorylated ERK1/2 expression (Fig. 8B, D), which could be attenuated by KMUP-1 (0.5 mg/kg/d) and sildenafil (0.7 mg/kg/d). KMUP-1 was more potent than sildenafil in inhibiting calcineurin A and ERK1/2 expression.

Discussion

We have recently shown that KMUP-1 attenuated isoprenaline-induced cardiac hypertrophy through the NO/cGMP pathway and signaling transduction in myocardium and cardiomyocytes [15]. This study further suggests that KMUP-1 can alleviate LVH induced by hypertension or L-NNA. In addition, we found that KMUP-1 restored eNOS in the aorta but downregulated iNOS in the left ventricle, both of which could mediate therapeutic effects on hypertensive LVH. Furthermore, we found that KMUP-1 had more marked suppressive effects on calcineurin A and ERK1/2 than sildenafil in hypertensive LVH.

In this study, increased eNOS and decreased iNOS expression are involved in the antihypertrophic effects of KMUP-1 in SHRs. This finding is consistent with the growing evidence that NO may have beneficial or deleterious effects on myocardium if derived from eNOS or iNOS, respectively [16,17]. NO stimulates cGMP synthesis through sGC activation, and the downstream cGMP-dependent PKG modulates cardiac remodeling and dysfunction [18]. NO formation and cGMP enhancement can improve the contractile function and sarcolemmal integrity of the heart [19]. Therefore, we assume that the cardioprotective effects conveyed by KMUP-1 in SHRs might be attributed to both increased eNOS and decreased iNOS expression. Precisely how KMUP-1 upregulates eNOS and inhibits iNOS in the hypertensive LVH is, however, not clear and warrants further studies.

We speculate that the mechanisms underlying the discrepant regulation of eNOS and iNOS by KMUP-1 are associated with its antioxidative and anti-inflammatory effects. It is well known that reactive oxygen species can decrease eNOS activity [20]. In addition, there is some...
evidence to show antioxidative effects conveyed by cGMP [21]. In fact, we have found that KMUP-1 increased eNOS activity and also had antioxidative activity. Accordingly, we speculate that, with its ability to enhance cGMP production, KMUP-1 might increase eNOS activity due to its antioxidant effect.

Regarding the suppressive effect of KMUP-1 on iNOS, our previous study showed that KMUP-1 was able to suppress the iNOS expression induced by tumor necrosis factor alpha (TNF-α) [13]. It is known that in a hypertensive status, there is increased production of cytokines such as TNF-α [22]. Therefore, it is possible that, in SHRs, the mechanisms by which KMUP-1 suppresses iNOS expression may be direct inhibition of the effects of TNF-α or indirect suppression of TNF-α production as a result of resolved hypertension.

Interestingly, even though in the present study the expression of eNOS was lower and the expression of iNOS higher in SHRs than WKY rats, there was no difference in the plasma levels of NOx between SHR and WKY rats. It is well known that iNOS-derived NO formation is markedly greater than that from eNOS. Nevertheless, it should be also noted that plasma NOx is an indirect indicator of the total endogenous production of NO from nonspecific tissue [23]. Therefore, it is possible that iNOS and eNOS, from either aorta or left ventricle, may both contribute to the production of plasma NOx. Accordingly, we assume that, in SHRs, NO production induced by upregulated iNOS may be offset by that induced by downregulated eNOS, with the net result of a slight tendency toward a higher but not significantly different NO production when compared with the levels in WKY rats.

Figure 7. Effect of KMUP-1 and sildenafil on protein kinase G (PKG) protein expression in the left ventricles of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. KMUP-1 and sildenafil significantly increased PKG expression. Each value represents the mean ± standard error (n = 6). (A) * p < 0.05, ** p < 0.01 versus the WKY-CTL group; p < 0.05 versus the SHR-CTL group; (B) ## p < 0.01 versus the SHR-CTL group; ** p < 0.01 versus the SHR-L-NNA group. CTL = control; L-NNA = N-omega-nitro-L-arginine.

A novel finding of the present study is that the antihypertrophic effects exerted by KMUP-1 are associated with regulation of eNOS and iNOS not only in the left ventricle, but also in the aorta. We speculate that KMUP-1 could affect vascular eNOS expression and that it first exerted antihypertensive effects [14]. Following this effect, KMUP-1 then improved LVH and subsequently reduced ventricular iNOS expression. In our study, the antihypertensive effect of KMUP-1 was markedly significant, so it could be caused by the vasodilatory action of KMUP-1 on enhancing endothelial NOS expression. Inhibition of PDE-5 by KMUP-1 can block the degradation of cGMP, thereby increasing its intracellular levels and evoking potent vasodilatory responses [12, 14]. The dual effects on both aorta and myocardium may play a predominant role of KMUP-1 in reducing hypertensive LVH. However, it should also be noted that KMUP-1 could lower the blood pressure in normotensive WKY rats even at low dosage, suggesting that hypotension might be a side effect of concern in future studies using higher doses.

ERK1/2 is activated in response to hypertrophic agonists and is itself a contributor to hypertrophy [24]. Calcineurin A has been reported to regulate the nuclear factor of activated T cells-dependent iNOS expression and cardiomyocyte protection [25]. In addition, calcineurin A can activate ERK1/2, and thus mediate cell proliferation and differentiation [26]. In our study, calcineurin A and ERK1/2 phosphorylation were increased in SHRs receiving L-NNA, and these effects were attenuated by both KMUP-1 and sildenafil, suggesting that they may also exert their cardioprotective properties through ERK1/2 and calcineurin A signaling pathways.

In summary, the present study indicates that KMUP-1 can improve hypertension-induced LVH and associated downregulation of eNOS in aorta and upregulation of iNOS in left ventricle [8,27]. In addition, its antihypertrophic effects are possibly mediated by signaling of calcineurin A and ERK1/2 pathways in the myocardium.
Acknowledgments
This work was supported by research grants NSC 95-2323-B-037-004 (Professor Ing-Jun Chen) and NSC-95-2314-B-037-089-MY3 (Professor Jiunn-Ren Wu) from the National Science Council of Taiwan, and by a grant from the Kaohsiung Medical University Hospital (KMUP99-9R24).

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