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The role of local and systemic renin angiotensin system activation in a genetic model of sympathetic hyperactivity-induced heart failure in mice


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Ferreira JC, Bacurau AV, Evangelista FS, Coelho MA, Oliveira EM, Casarini DE, Krieger JE, Brum PC. The role of local and systemic renin angiotensin system activation in a genetic model of sympathetic hyperactivity-induced heart failure in mice. Am J Physiol Regul Integr Comp Physiol 294: R26–R32, 2008. First published October 31, 2007; doi:10.1152/ajpregu.00424.2007.—Sympathetic hyperactivity (SH) and renin angiotensin system (RAS) activation are commonly associated with heart failure (HF), even though the relative contribution of these factors to the cardiac derangement is less understood. The role of SH on RAS components and its consequences for the HF were investigated in mice lacking α2A and α2C adrenoceptor knockout (α2A/α2CARKO) that present SH with evidence of HF by 7 mo of age. Cardiac and systemic RAS components and plasma norepinephrine (PN) levels were evaluated in male adult mice at 3 and 7 mo of age. In addition, cardiac morphometric analysis, collagen content, exercise tolerance, and hemodynamic assessments were made. At 3 mo, α2A/α2CARKO mice showed no signs of HF, while displaying elevated PN, activation of local and systemic RAS components, and increased cardiomyocyte width (16%) compared with wild-type mice (WT). In contrast, at 7 mo of age, α2A/α2CARKO mice presented clear signs of HF accompanied only by cardiac angiotensin II levels and increased collagen content (twofold). Consistent with this local activation of RAS, 8 wk of ANG II AT1 receptor blocker treatment restored cardiac structure and function comparable to the WT. Collectively, these data provide direct evidence that cardiac RAS activation plays a major role underlying the structural and functional abnormalities associated with a genetic SH-induced HF in mice.

sympathetic nervous system; AT1 receptor blocker; α2A/α2C adrenergic knockout mice

HEART FAILURE (HF) IS A PROGRESSIVE disorder that involves reduced cardiac output associated with neurohumoral changes, including activation of renin angiotensin (RAS) and sympathetic nervous systems (2, 4). The neurohumoral imbalance leads to cardiac dysfunction and hypertrophy, which culminates in cardiac remodeling and development of HF. Although the mechanisms for the transition of cardiac hypertrophy to HF are poorly understood, increased cardiac ANG II has been observed in a clinical course of human HF and related to a progressive impairment of ventricular function (39).

The RAS plays a prominent role in cardiac remodeling during HF development, since increased cardiac ANG II levels lead to cardiac myocyte hypertrophy and myocardial fibrosis (8, 21, 25). It is well known that all components required for ANG II production are available in the heart, and cardiac ANG II formation seems to be regulated independently of circulating RAS (17, 33, 43). Indeed, mechanical and humoral regulation of cardiac ANG II production in HF includes increased cardiac wall tension and sympathetic hyperactivity (9). The latter, by activating β-adrenergic receptors (β-AR), stimulates renin and angiotensinogen (Ao) synthesis in fibroblasts and Ao synthesis in cardiac myocytes. Despite evidence for positive feedback between β-AR system and RAS in producing cardiac pleiotropic peptides as ANG II (14, 21, 29), to date, there are no data on the role of sympathetic hyperactivity on cardiac RAS components in a setting of developing HF.

We previously reported that mice lacking both α2A/α2C adrenergic receptors (α2A/α2C ARKO) develop sympathetic hyperactivity-induced HF, which is related to cardiac dysfunction and remodeling with increased mortality rate at 7 mo of age (3, 34). In contrast, at 3 mo of age α2A/α2C, ARKO mice present preserved cardiac function with no significant mortality rate when compared with age-matched wild-type mice (27). Therefore, these mice provide a good model system for studying the influence of sympathetic hyperactivity on cardiac RAS activation in different stages of cardiac disease.

The present investigation was undertaken to establish the relationship of sympathetic hyperactivity and RAS components during the development of HF and the relative contribution of the latter to the cardiac structural and functional abnormalities observed in advanced stage of a genetic model of sympathetic hyperactivity-induced HF in mice.

METHODS

Study population. A cohort of male congenic α2A/α2CARKO mice in a C57BL6/J genetic background and their wild-type controls (WT) were studied at 3 and 7 mo of age. At 3 mo of age, α2A/α2CARKO mice display normal cardiac function (27), but at 7 mo of age, they...
CARDIAC ANGIOTENSIN II AND CARDIAC REMODELING INDUCED BY SYMPATHETIC HYPERACTIVITY

present severe cardiac dysfunction associated with exercise intolerance and increased mortality rate (3, 34). Mice were maintained in a 12:12-h light-dark cycle and temperature-controlled environment (22°C) with free access to standard laboratory chow (Nuvitual Nutrients S/A, Curitiba, PR Brazil) and tap water. This study was performed according to ethical principles in animal research adopted by Brazilian College of Animal Experimentation (www.cobea.org.br). In addition, this study was approved by the University of São Paulo Ethical Committee (CEP#059).

Graded treadmill exercise test. Exercise capacity, estimated by total distance run, is a widespread method of characterizing cardiac dysfunction in rodents and humans with HF (30, 34). Exercise tolerance was evaluated using a graded treadmill exercise protocol for mice as previously described (13). Briefly, after being adapted to treadmill exercises over a week (10 min of exercise session), mice were placed in the exercise streak and allowed to acclimatize for at least 30 min. Intensity of exercise was increased by 3 m/min (6–33 m/min) every 3 min at 0% grade until exhaustion.

Cardiovascular measurements. Heart rate and blood pressure were determined noninvasively using a computerized tail-cuff system (BP 2000 Visitech Systems) described elsewhere (19). Mice were acclimatized to the apparatus during daily sessions over 4 days, one week before starting the experimental period.

Structural analysis. All mice were killed and their tissues were harvested. Cardiac chambers were then fixed by immersion in 4% buffered formalin and embedded in paraffin for routine histologic processing. Sections (4 μm) were stained with hematoxylin and eosin for examination by light microscopy. Only nucleated cardiac myocytes from areas of transversely cut muscle fibers were included in the analysis. Quantification of left ventricular fibrosis was achieved by Sirius red staining. Cardiac myocyte width and ventricular fibrosis were measured in the left ventricle free wall with a computer-assisted morphometric system (Leica Quantimet 500, Cambridge, UK).

Immunohistochemistry. Cardiac samples for ANG II immunohistochemistry were obtained from WT and α2/α3-ARKO mice at 3 and 7 mo of age. Left ventricle sections were deparaffinized in xylene and rehydrated in ethanol. Retrieval of ANG II immunoreactivity was obtained by incubating sections in citrate buffer (0.01 M, pH 6.0) with further heating for 5 min in microwave oven. The presence of ANG II was evaluated in left ventricle tissue using anti-ANG II rabbit antiserum (1:400; Península, Belmont, CA) (15). The antigen was acclimatized to the apparatus during daily sessions over 4 days, one week before starting the experimental period.

Angiotensin-converting enzyme activity assay. Cardiac angiotensin-converting enzyme (ACE) activity was determined via fluorometric assay of cardiac tissue samples from 3- and 7-mo-old WT and α2/α3-ARKO mice, frozen in liquid nitrogen and stored at −70°C (1). Briefly, the homogenate supernatant was incubated for 30 min at 37°C with a fluorogenic substrate containing 10 μM Abz-FRK-(Dnp)P-OH (Abz is o-aminobenzoic acid; DnP is dinitrophenyl) in 0.1 M Tris buffer, 50 mM NaCl, and 10 mM ZnCl2, pH 7.0. Hydrolysis rate of the substrate hydrolyzed per minute, based on the calibration curve obtained from complete hydrolysis of each peptide.

Plasma renin activity assay. Plasma renin activity was determined in 3- and 7-mo-old WT and α2/α3-ARKO mice by radioimmunoassay using commercially available kits, according to the manufacturer’s instructions (Renin Kit, CISBIO International, Bagnoles, France).

Catecholamines measurements. Plasma noradrenaline was measured by HPLC using ion-pair reverse-phase chromatography coupled with electrochemical detection (0.5 V), as described by Monte et al. (28).

Immunoblot. Immunoblots of 3- and 7-mo-old WT and α2/α3-ARKO of heart and kidney homogenates were performed according to Towbin et al. (42). Briefly, liquid nitrogen frozen tissues were homogenized in a buffer containing 1 mM EDTA, 1 mM EGTA, 2 mM MgCl2, 5 mM KCl, 25 mM HEPES (pH 7.5), 100 μM PMSF, 2 mM DTT, 1% Triton X-100, and protease inhibitor cocktail (1:100, Sigma-Aldrich; St. Louis, MO). Samples were loaded and subjected to SDS-PAGE in polyacrylamide gels (10%). After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Amer sham Biosciences; Piscataway, NJ). Equal loading of samples (60 μg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blotted membrane. The blotted membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4°C with rabbit anti-human renin polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and sheep anti-human Ao antibody (1:200; a kind gift from Dr. S. Kumar, Farmington, UK). Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (anti-rabbit or anti-sheep, 1:5,000, for 1.5 h at room temperature) and developed using enhanced chemiluminescence (Amer sham Biosciences) detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion based on National Institutes of Health ImageJ). Targeted bands were normalized to renal GAPDH.

Real-time RT-PCR. RNA was isolated from heart tissue with TRIzol reagent (GIBCO, Invitrogen, Carlsbad, CA). RNA concentration and integrity were assessed; cDNA was synthesized using Superscript III RNase H-RT (Invitrogen) at 42°C for 50 min and real-time PCR was performed. The primers used for gene amplification were Ao sense, 5′-CCGGAGTCTCTACAATCTGC-3′; Ao antisense, 5′-TTTGATGGAGGGAGGTGTC-3′; Cyclophilin sense, 5′-AATGCTGGACCAAACACAAA-3′; and Cyclophilin antisense, 5′-CTTCCTTCTACCTTCCAAA-3′. Real-time PCR for Ao and housekeeper gene, cyclophilin were run separately, and amplifications were performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) by using SYBR Green PCR Master Mix (Applied Biosystems). The results were quantified as cycle threshold (Ct) values, where Ct is defined as the threshold cycle of the PCR at which the amplified product is first detected. Expression was normalized with cyclophilin levels as an endogenous reference.

ANG II AT1 receptor blocker. To test whether increased cardiac RAS would be functionally involved in ventricular dysfunction and remodeling of advanced-stage cardiomyopathy, α2/α3-ARKO mice were randomly assigned to receive either saline or ANG II AT1 receptor blocker (ARB, losartan, 10 mg/kg in drinking water) for 8 wk (from 5 to 7 mo of age). ARB did not change blood pressure, cardiac function, and structure of WT mice. Therefore, we used only one control WT group for further comparisons with α2/α3-ARKO groups.

Noninvasive ventricular function was assessed by two-dimensional guided M-mode echocardiography, in halothane-anesthetized WT and α2/α3-ARKO mice, before and after ARB treatment. Briefly, mice were positioned in the supine position with front paws wide open, and an ultrasound transmission gel was applied to the precordium. Transthoracic echocardiography was performed using an Acuson Sequoia model 512 echocardiograph equipped with a 14-MHz linear transducer. Left ventricle systolic function was estimated by fractional shortening as follows: fractional shortening (%) = [(LVEDD – LVESD)/LVEDD] × 100, where LVEDD means left ventricular end-diastolic dimension, and LVESD means left ventricular end-systolic dimension.

Cardiac myocyte width and ventricular collagen content after ARB treatment were measured in the left ventricle free wall, as previously described in Structural analysis.
Statistical analysis. All values are presented as means ± SE. Two-way ANOVA with a post hoc testing by Tukey was used to compare the effect of genotype (WT and α2A/α2C-ARKO mice) and cardiomyopathy stage (3 and 7 mo of age) on hemodynamics, cardiac structure, plasma noradrenaline, exercise tolerance, lung water retention, and RAS components. The relationship between cardiac Ao and ANG II levels was assessed by linear regression analysis. One-way ANOVA with post hoc testing by Tukey was used to compare the effect of treatment on fractional shortening (FS), LVESD, LVEDD, cardiomyocyte width, and cardiac fibrosis. Statistical significance was considered achieved when the value of P was <0.05.

RESULTS

Effect of sympathetic hyperactivity on hemodynamics, noradrenaline levels, and distance run. Physiological parameters of WT and α2A/α2C-ARKO mice are presented in Table 1. As expected, 3- and 7-mo-old α2A/α2C-ARKO mice presented higher plasma noradrenaline levels than age-matched WT mice. The increased noradrenaline levels were paralleled by resting tachycardia in 3- and 7-mo-old α2A/α2C-ARKO mice, while blood pressure remained unchanged when compared with age-matched WT mice. At 3 mo of age, distance run and lung wet/dry ratio of α2A/α2C-ARKO mice were similar to that observed in age-matched WT mice. In contrast, 7-mo-old α2A/α2C-ARKO displayed exercise intolerance and increased lung water retention when compared with age-matched WT mice. This result suggests that 7-mo-old α2A/α2C-ARKO mice present lung edema, which we previously demonstrated to be related to an impaired ventricular function (34).

Effect of chronic sympathetic hyperactivity on RAS components. It is well recognized that sympathetic hyperactivity activates other neurohumoral systems, such as RAS (26, 31). To test whether RAS activity is increased in α2A/α2C-ARKO, we evaluated cardiac ANG II in α2A/α2C-ARKO mice. As depicted in Fig. 1A, 3- and 7-mo-old α2A/α2C-ARKO mice displayed elevated cardiac ANG II levels when compared with age-matched WT mice. Inasmuch as ANG II induces myocyte hypertrophy, we evaluated cardiac myocyte cross-sectional diameter by quantitative morphometrical analysis. As shown in Fig. 1B, 3- and 7-mo-old α2A/α2C-ARKO mice displayed significantly greater cardiac myocyte cross-sectional diameter than age-matched WT mice. Of interest, cardiac myocyte hypertrophy in α2A/α2C-ARKO mice was paralleled by an increased ventricular fibrosis at 7 mo of age, represented by a twofold increase in cardiac collagen content when compared with age-matched WT mice (Fig. 1C). In contrast, cardiac collagen content of 3-mo-old α2A/α2C-ARKO mice remained unchanged when compared with age-matched WT mice (Fig. 1C).

To verify the RAS components involved in elevated cardiac ANG II in our genetic model of sympathetic hyperactivity-induced cardiomyopathy, we evaluated cardiac ACE activity, plasma renin activity, and kidney renin expression in α2A/α2C adrenoceptor knockout (ARKO) mice (solid bars). Note that α2A/α2C-ARKO mice presented elevated cardiac ANG II levels paralleled by an increased cardiac myocyte cross-sectional diameter. At 7 mo of age, α2A/α2C-ARKO mice presented ventricular fibrosis. Data are presented as means ± SE. *P < 0.05 vs. WT; †P < 0.05 vs. 3-mo-old mice.

Table 1. Physiological parameters in wild-type and α2A/α2C ARKO mice

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Data are presented as means ± SE. ARKO, α2A and α2C adrenoceptor knockout; HR, heart rate; BP, blood pressure; PN, plasma noradrenaline. *P < 0.05 vs. wild-type (WT); †P < 0.05 vs. 3 mo of age.

Fig. 1. Cardiac ANG II (A), cardiac myocyte width (B), and cardiac collagen volume fraction (C) in 3- and 7-mo-old wild-type (WT, open bars) and α2A and α2C adrenoceptor knockout (ARKO) mice (solid bars). Note that α2A/α2C-ARKO mice presented elevated cardiac ANG II levels paralleled by an increased cardiac myocyte cross-sectional diameter. At 7 mo of age, α2A/α2C-ARKO mice presented ventricular fibrosis. Data are presented as means ± SE. *P < 0.05 vs. WT; †P < 0.05 vs. 3-mo-old mice.
activities or renal renin expression levels. \(\alpha_2A/\alpha_2C\) ARKO mice rather displayed a similar cardiac ACE activity, decreased plasma renin activity, and renal renin expression when compared with age-matched WT mice (Fig. 2, A–C, respectively).

Because elevated cardiac ANG II in 7-mo-old \(\alpha_2A/\alpha_2C\) ARKO mice was not related to activity of cardiac ACE and plasma renin, we further investigated the cardiac Ao expression levels in \(\alpha_2A/\alpha_2C\) ARKO mice. As shown in Fig. 3, \(\alpha_2A/\alpha_2C\) ARKO mice displayed increased cardiac Ao gene (Fig. 3A) and protein (Fig. 3B) expression levels when compared with age-matched WT mice. These results suggest that increased cardiac Ao content is sufficient for cardiac ANG II formation in 7-mo-old \(\alpha_2A/\alpha_2C\) ARKO mice regardless reduced cardiac ACE and plasma renin activities. In fact, cardiac Ao levels presented a strong correlation with cardiac ANG II levels (Fig. 3C).

Effect of ARB treatment on left ventricle function and remodeling. ARB treatment was used to determine the relative contribution of RAS activation on cardiac derangements. As depicted in Fig. 4A, ARB treatment increased FS of \(\alpha_2A/\alpha_2C\) ARKO mice to WT values. The improved LV function was associated with a pronounced anticardiac remodeling effect, reducing cardiomyocyte width (Fig. 4B), cardiac collagen con-

Fig. 2. Cardiac angiotensin-converting enzyme activity (ACE, A), plasma renin activity (B) and kidney renin expression (C) in 3 and 7 mo-old wild-type (WT, open bars) and \(\alpha_2A/\alpha_2C\) ARKO mice (ARKO, solid bars). Note that 3-mo-old \(\alpha_2A/\alpha_2C\) ARKO mice presented elevated activity of cardiac ACE and plasma renin, and increased kidney renin expression. At 7 mo of age, \(\alpha_2A/\alpha_2C\) ARKO mice presented cardiac ACE activity similar to age-matched WT mice but decreased plasma renin activity and renal renin expression. Data are presented as means ± SE. *P < 0.05 vs. WT; †P < 0.05 vs. 7 mo of age.

Fig. 3. Cardiac angiotensinogen (Ao) gene (A) and protein (B) expression levels and correlation between cardiac angiotensinogen gene expression and cardiac ANG II levels (C) in 3 and 7 mo-old wild-type (WT, open bars) and \(\alpha_2A/\alpha_2C\) ARKO mice (ARKO, closed bars). Note that \(\alpha_2A/\alpha_2C\) ARKO mice presented increased cardiac Ao expression at both gene and protein levels, which presented a strong correlation with cardiac ANG II levels (r = 0.80, P < 0.05). Data are presented as means ± SE. *P < 0.05 vs. WT.
Sympathetic hyperactivity is a hallmark of progressive heart failure, and it has been associated with a poor prognosis (4, 12, 35). It is well known that increased sympathetic activity leads to activation of RAS (9, 36, 37), which ultimately worsens cardiac function and remodeling. In the present investigation, we used genetically modified mice based on the disruption of both α2A and α2C adrenoceptors to study the influence of sympathetic hyperactivity on the activation of RAS components during the progression of cardiac disease and the relative contribution of RAS to the cardiac structural and functional abnormalities observed in advanced-stage HF.

The main findings of the present study are that chronic sympathetic hyperactivity is associated with local RAS activation and that the deleterious cardiac effects can be counteracted by ARB treatment. At 3 mo of age, when α2A/α2CARKO mice have preserved exercise tolerance and ventricular function (27), the increased cardiac ANG II is related to increased activity of cardiac ACE and plasma renin, as well as an increased cardiac Ao expression. In contrast, at 7 mo of age, when α2A/α2CARKO mice display exercise intolerance, lung edema, and ventricular dysfunction, augmented cardiac ANG II levels rely on the increased cardiac Ao expression levels, since activity of cardiac ACE and plasma renin is decreased.

It has been previously demonstrated that β-AR stimulation leads to cardiac hypertrophy, which is related to induction of local RAS (5, 16, 18). In fact, independent cardiac RAS (10, 22) with all RAS components locally expressed (11, 22, 32) makes RAS more efficient to couple with adrenergic activation. Our data suggest that cardiac activation of RAS by sympathetic hyperactivity contributes to the increased cardiac ANG II levels in α2A/α2CARKO mice. This response was independent of an increase in cardiac afterload since blood pressure remained unchanged in α2A/α2CARKO mice.

As a result of increased cardiac ANG II levels, both 3- and 7-mo-old α2A/α2CARKO mice displayed cardiac myocyte hypertrophy. However, a ventricular fibrosis was restricted to 7-mo-old α2A/α2CARKO mice. ANG II is recognized for its hypertrophic and fibrosis effects (7). Our data suggest that at 3 mo of age, sympathetic hyperactivity leads to cardiac myocyte hypertrophy with no significant cardiac collagen deposition, which is related to preserved cardiac function and exercise tolerance, as previously reported (27). With the progression of cardiomyopathy, as observed in 7-mo-old α2A/α2CARKO mice, cardiac remodeling takes place with cardiac myocyte hypertrophy and myocardial fibrosis. The latter might involve both increased deposition and altered composition of extracellular matrix by ANG II via transforming growth factor and MAPKp42 and MAPKp44 signaling pathways, which have been previously demonstrated in ANG II-treated isolated fibroblasts (6).
The mechanism underlying the increased cardiac ANG II levels is related to disease stage. At 3 mo of age, α2A/α2C ARKO mice presented increased cardiac ACE activity associated with no changes in ACE gene expression (data not shown) and augmented plasma renin activity paralleled by an increased expression of renal renin. Thus, sympathetic hyperactivity per se (independent of cardiac remodeling and dysfunction) seems to increase cardiac ANG II levels by activating local RAS components (ACE) and circulating renin. In fact, we previously demonstrated that increased cardiac ACE activity is involved in isoproterenol-induced cardiac hypertrophy in rats (31). In addition, increased cardiac ACE has been also demonstrated in 4-mo-old proto-oncogene Vav3 knockout mice, which display increased sympathetic activity (37). At 7 mo of age, α2A/α2C ARKO mice present cardiac remodeling with increased fibrosis and cardiac myocyte hypertrophy associated with a further increase in plasma noradrenaline (see Table I for details) and increased cardiac ANG II. The increased local ANG II occurred even with decreased local ACE and plasma renin activities, which suggests that other mechanisms are taking place in the augmented cardiac ANG II levels. Our data corroborate that of Heller et al. (17), who found a positive correlation between plasma renin activity and cardiac hypertrophy at 3 days of pressure overload in rats but not at 42 days. It is also important to highlight the existence of an autocrine/paracrine RAS (9), which might contribute to the increase in cardiac ANG II levels in 7-mo-old α2A/α2C ARKO mice. Recent reports have demonstrated that cardiac mast cells express renin at mRNA and protein levels (20). When mast cell degranulation is induced by hyperadrenergic dysfunction, released renin converts Ao into ANG I (40), and further local increase of ANG II triggers cardiac arrhythmias (23).

Although circulating levels of Ao have not been assessed, we speculate that the increase in cardiac ANG II levels are secondary to augmentation of Ao expression levels, which may indicate that this is a rate-limiting step for the local increase of ANG II levels in advanced-stage cardiomyopathy. Indeed, there was a strong correlation between cardiac Ao expression and cardiac ANG II, and the ARB treatment counteracted the cardiac deleterious effects. Furthermore, under normal conditions, low levels of cardiac Ao mRNA and protein levels are observed (38), which are promptly increased upon cardiac disease states (24, 41).

In summary, α2A/α2C ARKO mice at 3 mo of age presented local and systemic activation of RAS associated with no signs of HF but increased cardiomyocyte width. In contrast, at 7 mo, when advanced stage of cardiomyopathy ensued, we detected only cardiac increases in expression of Ao and ANG II levels. The cardiac structural and functional abnormalities were counteracted by ARB treatment underscoring the role of local RAS activation in this process. Taken together, we provided direct evidence that local RAS activation plays a role in a genetic model of sympathetic hyperactivity-induced HF in mice.

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GRANTS

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