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βγ-CAT, Induced Isolated Rabbit Thoracic Aortic Rings Sustained Contraction in Endothelial-dependent Manner

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Abstract: In vertebrates, non-lens βγ-crystallins are widely expressed in various tissues and their functions are not well known. The molecular mechanisms of trefoil factors (TFFs), which involved in mucosal healing and tumorigenesis, have remained elusive. βγ-CAT is a novel multifunctional protein complex of non-lens βγ-crystallin and trefoil factor from frog skin secretions. Here we report that βγ-CAT could induce sustained contraction of isolated rabbit aortic rings in dosage (2-35 nmol/L) and endothelium dependent manners (P<0.01). In addition, *in situ* immunofluorescence indicated that positive TNF-α signals were mainly detected at the endothelial cell layer of βγ-CAT (25 nmol/L) treated rings. Furthermore，βγ-CAT induced primary cultured rabbit thoracic aortic endothelial cells (RAECs) rapidly to release TNF-α. After βγ-CAT (25 nmol/L) treated for 10 and 30 min, the levels of the endothelial cells released TNF-α were 34.17±5.10 pg/mL and 98.01±4.67 pg/mL (P<0.01), respectively. In conclusion, βγ-CAT could induce sustained contraction of isolated aortic rings, and the contractile effect might be partially explained by the release of TNF-α. These findings will give new insight into understanding the functions and physiological roles of non-lens βγ-crystallins and trefoil factors.

Key words: Tumor necrosis factor-α; Trefoil factor; Non-lens βγ-crystallin; Endothelium-dependent aorta vasoconstriction; βγ-CAT

非晶状体βγ晶状体蛋白与三叶因子蛋白复合物诱导兔胸主动脉环内皮依赖的持续收缩效应

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摘要：脊椎动物中，非晶状体βγ–晶状体蛋白广泛分布于各种组织，但是功能知之甚少。三叶因子在创伤修复与肿瘤发生中具有重要作用，其分子作用机制尚不清楚。非晶状体βγ晶状体蛋白与三叶因子蛋白复合物(βγ-CAT)是一个从大蹼铃蟾皮肤分泌物中分离的一类全新的蛋白复合物。研究表明，βγ-CAT 能够诱导离体的兔胸主动脉产生快速而持续的收缩，结合药理学抑制剂，细胞培养，激光共聚焦显微镜和免疫荧光原位组化，从细胞和分子水平对其作用机制进行研究。结果表明：βγ-CAT 诱导兔胸主动脉产生的收缩效应为剂量依赖(2—35 nmol/L)和内皮依赖(P<0.01)。在βγ-CAT(25 nmol/L)处理的主动脉环的内皮细胞层检测到肿瘤坏死因子-α的释放。同时，βγ-CAT 能够诱导原代培养的兔胸主动脉内皮细胞(RAEC)快速释放肿瘤坏死因子-α，βγ-CAT(25 nmol/L)分别处理 5 和 30 min，RAEC 释放的肿瘤坏死因子-α的浓度分别为(34.17±5.10)pg/mL 和(98.01±4.67) pg/mL (P<0.01)。表明肿瘤坏死因子-α在βγ-CAT 诱导兔胸主动脉产生的收缩效应中发挥重要作用。为进一步深入研究非晶状体βγ晶状体蛋白与三叶因子的生理功能提供了新的思路和线索。

关键词：肿瘤坏死因子-α；三叶因子；非晶状体βγ–晶状体蛋白；非晶状体βγ晶状体蛋白与三叶因子蛋白复合物；内皮

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依赖的主动脉收缩

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Crystallins are abundant proteins found in the eye lens of vertebrates, which contain two super families, α-crystallins and βγ-crystallins (Wistow & Piatigorsky, 1988; Bhat, 2004). βγ-crystallins, which contain repeats of a characteristic Greek key motif consisting of about 40 amino acidic residues, are thought to play a structural role in the mammalian eye lens, but their non-structural functions, which appear to be very important, have not been elucidated (Wistow & Piatigorsky, 1988; Bhat, 2004). Up to now, a lot of non-lens βγ-crystallins were discovered in microbes and mammals, such as microbial stress-inducible Protein S (Nelson & Zusman, 1983) and Spherulin 3a (Nelson & Zusman, 1983; Rosinke et al, 1997), epidermis differentiation-specific proteins, also named ep37 in amphibians (Takabatake et al, 1992; Wistow et al, 1995; Ogawa et al, 1997) and absent in melanoma 1(AIM1) in mammals (Ray et al, 1997; Teichmann et al, 1998). Ep37 proteins were found in embryonic epidermis, cutaneous glands and gastric epithelial cells of the amphibian *Cynops pyrrhogaster* and have been proposed to participate in epidermis development (Ogawa et al, 1997; Ogawa et al, 1998). The different size of AIM1 mRNAs transcripts are temporally regulated during embryogenesis and also found in adult skin, heart, lung and liver, and proposed to have a tumor suppression function (Ray et al, 1997; Teichmann et al, 1998). However, little information is known about the biochemical properties, functions and action mechanisms of these non-lens βγ-crystallins in vertebrates.

The trefoil factor (TFF) proteins are secreted proteins that are characterized by a conserved motif known as the TFF domain (or P-domain previously), which consists of about 40 amino acidic residues (Sands & Podolsky, 1996; Thim, 1997). In amphibians, a single TFF-domain (xP1), two TFF-domain (xP2, Bm-TFF2), three TFF-domain (β-sununit of βγ-CAT) and four TFF-domain (xP4) peptides were discovered (Hauser & Hoffmann, 1991; Hauser et al, 1992; Zhang et al, 2005; Liu et al, 2008). In mammals, there are three closely related known TFF peptides. TFF1 and TFF3 contain a single TFF domain, while TFF2 contains two TFF domains (Thim & May, 2005). It is well known that TFFs mainly expressed in specific epithelial cells of the gastrointestinal tract and played an important role in the gastrointestinal tract repair and healing by stimulating the migration of cells at the mucosal wounding edges (Lefebvre et al, 1996; Taupin & Podolsky, 2003). In addition, TFFs may play a possible role in immune regulation as a potential inflammatory mediator (Taupin & Podolsky, 2003; Baus-Loncar et al, 2005). It was reported that serum concentrations of TFF1, TFF2, and TFF3 were significantly increased (200 pmol/L) in inflammatory bowel disease patients (Gronbaek et al, 2006). The answers to the key questions, such as whether a receptor mediates the functional effects of TFFs, will offer an insight into understanding the molecular mechanisms involved in TFFs actions (Taupin & Podolsky, 2003).

The Chinese red belly frog (*Bombina maxima*) is an endemic amphibian living in the mountainous regions of southwestern China. It is known by the indigenous people that the frog lives in very harsh environments and its skin secretion is very toxic. βγ-CAT is the first example of a naturally existing protein complex of a non-lens βγ-crystallin and a trefoil factor identified from Chinese red belly frog (*Bombina maxima*) skin secretions (Liu et al, 2008), which is responsible for the potent hemolytic activity of the secretions on mammals. The α- and β-subunits of βγ-CAT, with a non-covalently linked form of $\alpha\beta_2$, shows significant sequence similarity to ep37 proteins, which are a group of non-lens βγ-crystallins characterized in newt *Cynops pyrrhogaster,* and frog *Xenopus laevis* trefoil factors, respectively. βγ-CAT was a multiple-functional protein. βγ-CAT caused some mammalian erythrocytes hemolytic via membrane pore formation (Liu et al, 2008). In human umbilical vein endothelial cells (HUVECs), βγ-CAT was rapidly endocytosed via intracellular vacuole formation, translocated into the cell nucleus, and then regulated cell migration and wound healing, survival and apoptosis, depending on the dosages used(Liu et al, 2008). Through Western blotting analysis, we found that the α- and β-subunits of βγ-CAT participated in a 150 kDa SDS-stable protein complex formation during the process of βγ-CAT endocytosis, which also contained positive ubiquitination signals. Furthermore, the immunofluorescence signals of ubiquitin and βγ-CAT subunits were co-localized in the vacuoles that were distributed in the cytoplasm and nucleus (He et al, 2008b). In whole animal level, βγ-CAT showed potent lethal toxicity on mammals resulted from hypotension

and cardiac inhibition (Qian et al, 2008a). In isolated rabbit heart Langendorff preparations, βγ-CAT elicited an acute negative inotropic effect on isolated rabbit hearts, which mimicked acute heart failure and was endothelium-dependently. The rapid and significant release of tumor necrosis factor-α (TNF-α) was detected both *in situ* of the organ and in primary cultured rabbit endocardial endothelial cells (REECs), which might be partially responsible for the myocardial depression caused by βγ-CAT (Qian et al, 2008b).

Here, we report that βγ-CAT could induce isolated rabbit thoracic aortic ring contraction in dosage and endothelium dependent manners.

1 Materials and Methods

1.1 Animals and ethics

Male New Zealand White rabbits were purchased from the Experimental Animal Center of Kunming Medical College. The care of experimental animals and all experimental handling procedures were in accordance with the Chinese animal protection laws and officially approved guidelines by the Ethics Committee of Kunming Institute of Zoology, the Chinese Academy of Science.

1.2 Purification of βγ-CAT

The procedure of βγ-CAT purification followed a method described previously (Liu et al, 2008). Briefly, the lyophilized frog *B. maxima* skin secretions (from a stock in the Kunming Institute of Zoology) was dissolved in 10 mL 50 mmol/L Tris-HCl buffer, pH 7.3, containing 5 mol/L EDTA, dialyzed against the same buffer at 4 ℃ overnight. After centrifugation, the supernatant was loaded on a series of DEAE Sephadex A-50 (Pharmacia) column, Sephadex G-100 (Pharmacia, superfine) column and AKTA Mono-Q HR5/5 anion ion exchange column and eluted as described previously. Protein concentration was determined by a protein assay kit (Bio-Rad, CA, USA) with BSA as a standard.

1.3 Contractile response of rabbit thoracic aorta ring *in vitro*

The isolated thoracic aortic ring contractile activities induced by βγ-CAT *in vitro* were detected as previously described (Pfister & Campbell, 1992). Briefly, the male New Zealand White rabbits (weight about 2.5-3.0 kg) were anesthetized with 20% urethane by ear marginal vein injection. Segments of the intact thoracic aorta were carefully isolated and excessive fat and connective tissues were removed, then aortic rings were immediately immersed in ice-cold Krebs-Henseleit buffer solution (KHBS, containing 125 mmol/L NaCl, 4.3 mmol/L KCl, 1.1 mmol/L KH_2PO_4, 1.3 mmol/L $MgCl_2$, 2.4 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$ and 13.32 mmol/L glucose). The aorta was cut into 5 mm-width rings without touching the luminal surface. The aortic rings were placed in bathcups, which contained 20.0 mL oxygenated (95% O_2 and 5% CO_2) KHBS kept at 37°C with a thermostatic circulator. One end of the ring was mounted vertically in a force transducer and the other end connected to a barb. The tissues were preloaded with a resting tension of 1.0 g and equilibrated for 1 h before being exposed to drugs. Immediately before the assay, the contractility of the aortic ring was checked by 60 mmol/L KCl. To construct the concentration-response curves of βγ-CAT, various dosages of βγ-CAT were administered to aortic rings in a cumulative manner. The contractile effect was expressed as a percentage of the maximum force of 60 mmol/L KCl developed on the aortic ring. The buffer solution (20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.8) of βγ-CAT was used as a negative control. The tissue's contractile tension was recorded and analysed by using MedLab software package (Nanjing Medease Technology Development Co. Ltd).

1.4 Effect of endothelium-dependent contraction

To investigate the role of endothelia in the contraction, the endothelia of thoracic aorta were tenderly rubbed with a moistened cotton swab. This rubbing procedure was reported to remove at least 95% of the endothelia (Furchgott & Vanhoutte, 1989). The relaxant response of acetylcholine (3 μmol/L, Sigma) to Phenylephrine (3 μmol/L, Sigma) induced aortic contraction, and was used to evaluate the extent of endothelium removal after rubbing. The endothelium-removed aortic rings were equilibrated for 1h in KHBS and treated with βγ-CAT (fixed at 25.0 nmol/L). The endothelium-intact rings were used as a control.

1.5 Cell culture

The primary culture of rabbit thoracic aortic endothelium cells (RAEC) was performed using the modified methods described previously (Pomerat & Slick, 1963; Buonassisi & Venter, 1976). Briefly, male New Zealand rabbits were anesthetized with 20% urethane by ear marginal vein injection and the intact thoracic aorta was carefully isolated. The intact thoracic aorta was rinsed with sterile PBS (containing 100 U/mL penicillin and 100 μg/mL streptomycin) three times. The inner side of the thoracic aorta was injected with 3 mL 0.1% collagenase (type IA, Sigma) in PBS at 37℃ for

20 min. Flushed with 30 mL sterile PBS and 10 mL DMEM medium with 10% FBS (Gibco), the cells were collected by centrifugation at 1000r/min for 10min. The pellet was re-suspended in DMEM medium (containing 10% FBS, 2mmol/L L-glutamine, 100U/mL penicillin, 100 μg/mL streptomycin) and grown in an incubator filled with 5% CO_2 at 37℃. Confluent cells were subcultured using trypsin/EDTA to subsequent flasks. The purity of cultured cells was analyzed by staining with Alexa Fluor 594 AcLDL (Molecular Probes). The confluent RAECs used in the experiments were taken from the second to the fourth passages that the purity was higher than 95%.

1.6 Cell viability assay

Cell viability was detected using the MTT method as described previously (Alley et al, 1988). Briefly, various doses of βγ-CAT were added to the confluent RAEC cells (5×10^4 cell/well) in 96-well plates and incubated for 2h at 37℃, 5% CO_2. Then the medium was aspirated off and replaced with fresh medium containing MTT. Incubated for 4 h, the MTT-containing medium was removed. DMSO extraction was added and the micro titer was read at 595 nm. Cell viability was determined by comparing the test wells values with the positive and negative control. Each dose was replicated three times.

1.7 TNF-α releasing assay

The concentrations of cells released TNF-α induced by βγ-CAT were determined by ELISA kit (Biosource, Camarillo, California). Briefly, the confluent RAECs (5×10^4 cell/well) had been plated onto 96-well plates and treated with βγ-CAT (5 nmol/L and 25 nmol/L) for different time (10 and 30 min).The supernatant was obtained by centrifugation. The concentrations of released TNF-α in the supernatant were determined by measuring absorbance at 450 nm using an ELISA reader and calculated from a standard curve.

1.8 Immunohistochemistry

The rabbit aortic rings were pre-treated with βγ-CAT (25 nmol/L) to provoke sustained contraction, and then the rings were frozen immediately in liquid nitrogen and embedded with Tissue-Tek O.C.T. (Sakura Finetek, USA). The tissue was cut into 10 μm thick sections and mounted on poly-L-lysine coated slides, then fixed with ice-cold acetone. The released TNF-α in the rabbit aortic ring sections was detected with mouse monoclonal anti-TNF-α antibody and conjugated-FITC goat anti-mouse antibodies (Santa Cruz). The nucleus was counter-stained with PI. The sections were observed under a confocal microscope (LSM510 META, Zeiss, Plan-Neofluar 20×/0.5).

1.9 Statistical Analysis

Experimental values expressed as means±SE. Statistical significance was tested by student's *t*-test for one-way analysis of variance. The level of statistical significance was set at the level of $P<0.05$.

2 Results

2.1 Contractile response to βγ-CAT

Fig. 1A indicated that purified βγ-CAT could induce sustained contraction of isolated rabbit aortic rings in a concentration-dependent manner. When the concentration of βγ-CAT was increased to 30.0 nmol/L, the contractile response reached 102.40±6.56% of the maximum contraction induced by 60mmol/L KCl (n=6, Fig. 1B). Even treated with 35 nmol/L βγ-CAT, the contractile response was still at a plateau, which equaled to 100.30±3.45% of the maximum contraction induced by 60mmol/L KCl. Based on the dose response curve, the EC_{50} of βγ-CAT contractile effect was estimated to be approximately 10 nmol/L (Fig 1.B).

2.2 Endothelium-dependent contractile response to βγ-CAT

Fig. 2A showed that in endothelium-intact aortic rings, βγ-CAT induced the contractile effect of 87.97±5.21% equal to the maximum contraction induced by 60 mmol/L KCl, while the endothelium-removed aortic rings produced only 18.88±4.7% (n=30, $P<0.01$, Fig. 2A, B). This result indicated that the βγ-CAT induced rabbit aortic ring contraction is endothelium-dependent.

2.3 Cell viability

After the βγ-CAT treated for 2h, the viability of primary cultured RAECs was decreased gradually in a dose-dependent manner. In the 5 nmol/L βγ-CAT treatment, cell viability was slightly decreased to 93.11±5.12% (n=6). When treated with 25 nmol/L βγ-CAT, cell viability was decreased to 87.93±8.62% (n=6). Cell viability was decreased to 49.75±6.79 % (n=6, $P<0.01$, compared with the negative control) after 100 nmol/L βγ-CAT treatment.

2.4 Detected the releasing TNF-α from βγ-CAT treated aorta rings *in situ*

As shown in Fig. 3A, in the βγ-CAT treated aortic rings sections (right panels), the positive TNF-α signals was mainly distributed at the endothelial cell layer (marked by arrows). In the control tissue sections (left panels), no obvious positive signals were detected.

2.5 βγ-CAT induce RAEC release TNF-α

Fig. 3B indicated that the level of TNF-α released from βγ-CAT treated cells was increased in a concentration-dependent manner. In basal conditions, the TNF-α level in the supernatant of RAECs was very low. The concentrations of TNF-α were 11.35±3.45 pg/mL and 23.50±6.24 pg/mL (n=6) at 10 and 30 min points, respectively. In 5 nmol/L βγ-CAT treated cells, the TNF-

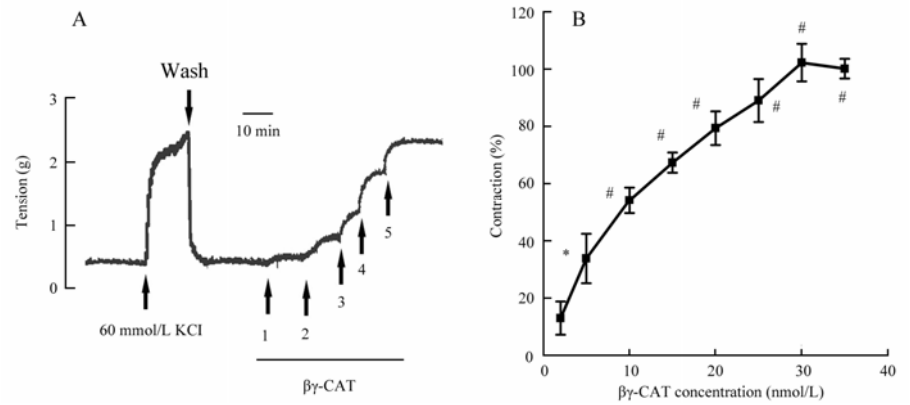

Fig. 1 βγ-CAT induced contractile effect on isolated rabbit aortic rings

A: βγ-CAT induced sustained contraction of isolated rabbit aortic rings. Endothelium-intact rabbit aortic rings were prepared as described in the material and methods. Contraction induced by 60 mmol/L KCl was used for reference (left part). βγ-CAT was added to the organ bath with several concentrations (right part): (1) 2 nmol/L; (2) 5 nmol/L; (3) 10 nmol/L; (4) 20 nmol/L; (5) 30 nmol/L. B: Dose-response curve of βγ-CAT induced isolated rabbit aortic ring contraction. Contractile effect was expressed as a percentage of the maximum contraction induced by 60 mmol/L KCl. Data presented as mean ± SE (n=6, n refer to the number of rabbit from which the aortas were taken). * present P<0.05, # present P<0.01, compared with those in the control.

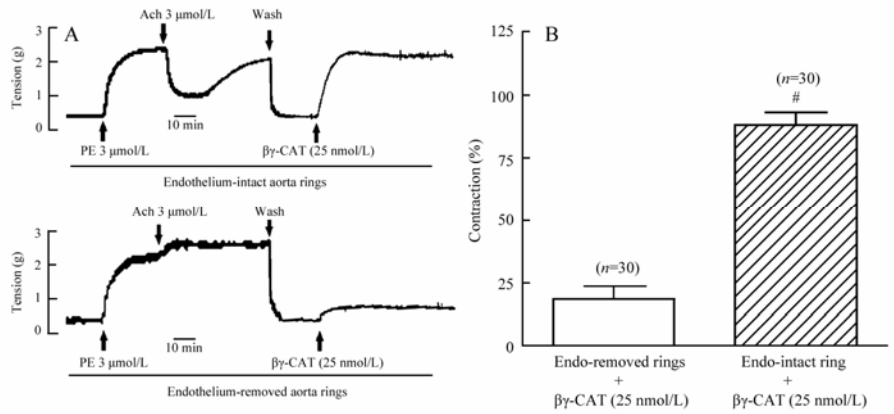

Fig. 2 βγ-CAT induced endothelium-dependent contractile effect

A: Compared the contractile response of the endothelium-intact and endothelium-removed rings to βγ-CAT. Endothelium-intact and endothelium-removed aortic rings were prepared as described in the material and methods. The relaxant response of acetylcholine (Ach, 3 μmol/L, Sigma) to Phenylephrine (PE, 3 μmol/L, Sigma) induced aortic contraction was used to evaluate the extent of endothelium removal after rubbing. The concentration of βγ-CAT was fixed at 25 nmol/L and the contractile responses of the endothelium-intact (upper) and endothelium-removed aortic rings (lower) to βγ-CAT were compared. B: Statistical analysis of the contribution of endothelium to the βγ-CAT induced contraction. Contractile effect was expressed as a percentage of the maximum contraction induced by 60 mmol/L KCl. Data presented as means±SE (n=30, n refer to the number of rabbit from which the aortas were taken). # present P<0.01, compared with the control.

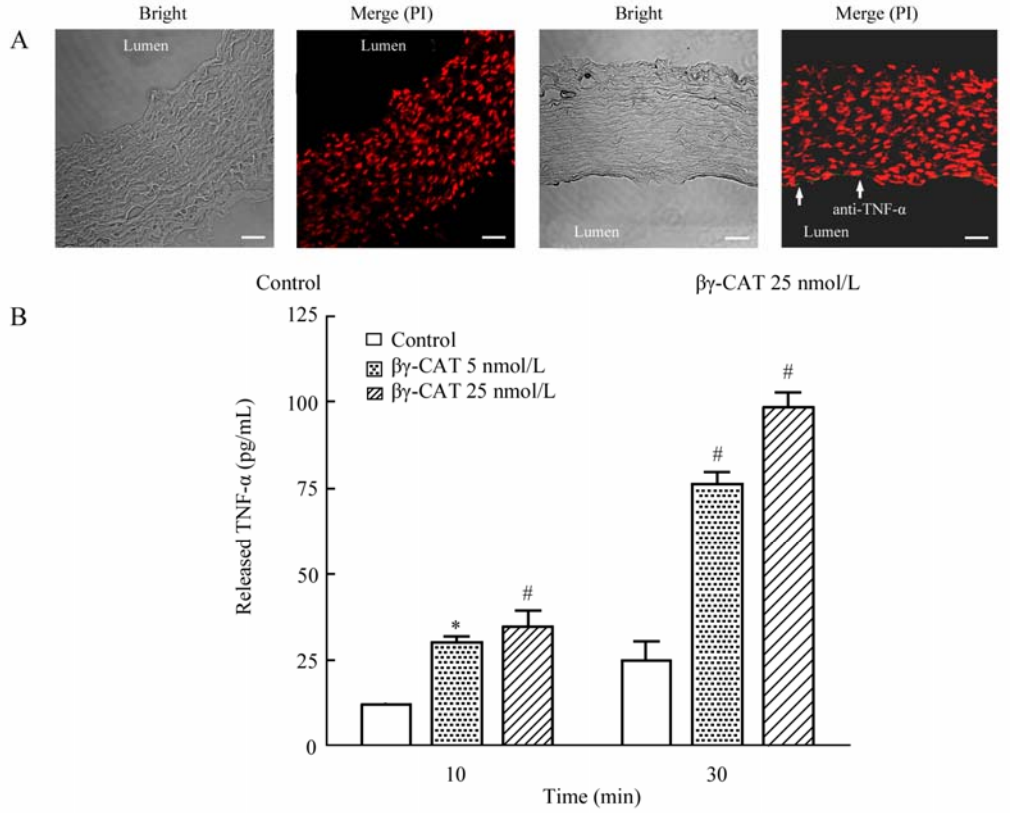

Fig. 3 Detection of the release of TNF-α

A: The released TNF-α from βγ-CAT treated aortic ring was detected *in situ* by indirect immunofluorescence. Nucleus was counter-stained by PI (red channel). The control (left panels) and βγ-CAT treated tissues (right panels) were labeled with a specific antibody against TNF-α (green channel). The positive TNF-α signals are marked by arrows. Scale bars equal to 50 μm. B: Detecting released TNF-α from βγ-CAT treated RAECs through an ELISA kit. PBS treated used as the negative control. Data presented as means ± SE (n=6). * present P<0.05, # present P<0.01, compared with those in the control.

α levels were increased to 29.50±2.34 pg/mL (n=6, P<0.05, compared with the negative control) and 75.67±4.10 pg/mL (n=6, P<0.01, compared with the negative control) at 10 and 30 min points, respectively. After the cells were treated with 25 nmol/L βγ-CAT, the level of released TNF-α was increased to 34.17±5.10 pg/mL (n=6, P<0.05, compared with the negative control) and 98.00±4.67 pg/mL (n=6, P<0.01, compared with the negative control) at 10 and 30 min points, respectively.

3 Discussion

Our results indicated that βγ-CAT could induce sustained contraction of isolated rabbit aortic rings in a concentration dependent manner. This suggests that βγ-CAT can directly influence the vascular contractility and increase the tonicity of blood vessels. A variety of mechanisms are known to regulate vascular contractility, but systematic nervous and hormonal factors and factors from platelets were excluded from this experimental condition. Therefore, the possible mechanisms for the instant aortic rings sustained contractile effect induced by βγ-CAT are: (1) to directly work on the vascular smooth muscle and increase contractility, such as by interacting with the histaminergic receptors (Bevan et al, 1975), muscarinic receptors, the adrenergic receptors (Christ, 1995) or the serotonin receptors (Yokoyama et al, 1983) in the aortic ring to induce the vasoconstriction of the rings; and (2) maybe act on the endothelial cells and indirectly regulate the vascular contractility, which could produce endothelium-derived contracting factors, such as nitric oxide, vasoconstrictor peptides (angiotensin II, endothelin-1), arachidonic acid and inflammation cytokines (TNF-α), and contribute to initiating contraction of the vascular smooth muscle cells that surround them (Furchgott & Vanhoutte, 1989; Rubanyi, 1991; Vanhoutte et al, 2005). In our early investigation, some antagonists, including atropine, chlorpheniramine, phentolamine and S006 (Ketanserin tartrate salt), were used to investigate the vascocontraction effect induced by βγ-CAT. The endothelium-intact thoracic aorta was

prepared as described previously, and βγ-CAT dosage was fixed at 25 nmol/L. Three assay models were employed to investigate the interaction between βγ-CAT and antagonists: 1) the rings were pretreated with antagonists, 1 min later βγ-CAT was added; 2) sustained contraction was induced to the rings by βγ-CAT, then the antagonists were added; 3) the rings were treated with antagonists and βγ-CAT simultaneously. The histamine, 5-HT, noradrenaline and acetylcholine (Ach) were used as the positive control. Even the concentration of antagonists reached to 30μmol/L, which was about 1000 times higher than βγ-CAT, but the contractile effect of rabbit aortic rings induced by βγ-CAT was not noticeably affected. In some conditions, the contractile effects induced by acetylcholine, epinephrine and 5-HT were completely abolished (Data not shown). These results suggested that the vascocontractile activity of βγ-CAT is less likely to be due to the former possibility.

Vascular endothelium plays a pivotal role in modulation of vascular tone by producing vasoconstrictors, such as thromboxane A2 and endothelin (ET)-1, and vasodilators, such as prostacyclin and endothelium-derived relaxing factor (Furchgott & Vanhoutte, 1989). It is well known that endothelial cells (ECs) activation is the key event associated with acute and chronic inflammation (Cines et al, 1998). Activated ECs express new proteins and secrete chemokines, regulating the inflammatory response. Pro-inflammatory cytokines also affect vascular function and endothelium-derived factors are involved in regulation of blood pressure. Tumor necrosis factor-alpha (TNF-) is an important pro-inflammatory cytokine and has been shown to induce structural as well as functional alterations in endothelial cells (Conrad & Benyo, 1997; Giardina et al, 2002; Granger, 2006). TNF-α not only enhances vascular contraction, but also affects the ability of vascular endothelial functions to release various chemical mediators (Giardina et al, 2002). In this research, after comparing the vascocontraction effect of βγ-CAT between the endothelium-intact and endothelium-removed aortic rings, we found that the contractile effect of βγ-CAT was significantly endothelium-dependent (Fig. 2, $P<0.01$). In addition, we found that the released TNF-α was mainly distributed at the coronary endothelial cell layer of βγ-CAT treated aortic ring sections (Fig. 3A). Furthermore, the rapid and significant release of TNF-α was also detected in the supernatant of βγ-CAT treated RAECs (Fig. 3B). Previous research reported that βγ-CAT could be rapidly endocytosed via intracellular vacuole formation and translocated to human umbilical vein endothelial cells nuclei (Liu et al, 2008). Microarray analysis showed that βγ-CAT significantly up-related expression of a number of inflammation related cytokines, such as the tumor necrosis factor, interleukin 1β, interleukin 6 (Liu et al, 2008), which induced greater inhibition of vascular relaxation and enhancement of contraction in systemic vessels as one of the potential mediators of increased vascular resistance (Granger, 2004). These cytokines are in accordance with inflammatory mediation and immune regulation roles of trefoil factors (Taupin & Podolsky, 2003; Baus-Loncar et al, 2005; Giraud et al, 2007). In previous research, we reported that βγ-CAT could endothelium-dependently elicite an acute negative inotropic effect on isolated rabbit hearts by significant release of tumor necrosis factor-α from endocardial endothelial cells, which mimicked acute heart failure (Qian et al, 2008b). Taken together, these results suggest that TNF-α, released from the endothelia induced by βγ-CAT, could mediate the vasocontractile effect in a paracrine manner. It may at least partly explain the aortic rings contractile effect induced by βγ-CAT.

Some pore-forming toxins, such as Staphylococcal alpha-toxin (Wurzel et al, 1966; Buerke et al, 2002), botulinolysin (Sugimoto et al, 1995), lysenin (Sekizawa et al, 1996) and Equinatoxin II (Drevensek et al, 2002), could induce contractility of aortic rings in endothelium dependent or independent manners, as βγ-CAT is able to cause mammalian erythrocyte hemolysis via membrane pore-formation and release of cellular potassium (Liu et al, 2008). It suggests that the sustained contractile effect of aortic rings induced by βγ-CAT may be also mediated via cell membrane damage and intracellular potassium release.

Since in mammals, non-lens βγ-crystallins are widely expressed in various tissues and it was proposed that they were involved in epidermis development and tumor suppression (Trent et al, 1990; Wistow et al, 1995; Ray et al, 1996; Ray et al, 1997; Teichmann et al, 1998). TFFs have been shown to interfere with crucial biological processes such as cell proliferation, differentiation, apoptosis and angiogenesis, and there is convincing evidence that TFFs do play an important role in tumorigenesis (Rio et al, 1988; Lefebvre et al, 1996; Taupin & Podolsky, 2003). However, little information is known about the properties, functions and molecular mechanisms of these proteins.

Erspamer predicted that every protein and peptide

present in frog skin, sometimes in very large amounts, will be found in mammals, a prediction that is well supported by cumulative experimental evidence (Bevins & Zasloff, 1990). In many instances, results obtained from the study of amphibian skin components can serve as a guide for investigating the possible occurrence of identical or similar molecules and their action mechanisms in mammals, substantially contributing to the understanding and interpretation of facts assessed in higher vertebrates. We should express many thanks to this little lovely frog, which provide us an enlarged model, for the first time, to find the functional linkage of non-lens βγ-crystallins and trefoil factors. βγ-CAT is the first example of a naturally existing complex of non-lens βγ-crystallin and trefoil factor identified from frog *B. maxima* skin secretions. The distribution of βγ-CAT and the homologues of its each subunit in frog skin were observed in intestine and stomach, around the epidermis and granular glands imbedded in the dermis of the frog (Liu et al, 2008). Frog skin is naked and constantly confronted by a complex mixture of potentially injurious mechanical and chemical factors. Constant skin renewal and repair in disruptions of the surface layer of cells occur frequently. It is reasonable to hypothesize that rich distribution of such proteins in frog skin, especially in epidermis, could play important physiological roles in skin tissue repair and maintaining of tissue homeostasis.

In our published research, we also found that βγ-CAT could induce several tumor cell's detachment and apoptosis, and selectively kill tumor cells, such as HCT116，HT29 and A375 (He et al, 2008b). In addition, βγ-CAT triggered two types of cellular responses in human melanoma A375 cells. On one hand, βγ-CAT (25−200 pmol/L) could stimulate A375 cells migration. On the other hand, it inhibited A375 cells proliferation by delaying S-G2/M cell phase transition in cell cycle, associating with significantly increased the expression of p21/WAF1 and JunB, which are well-known cell proliferation inhibitor, and reduced the expression amounts of Cdc2 and Cdc25C (He et al, 2008a). Furthermore, it contains conserved Walker B motifs (IILYDEPS, residues 6-13) and Walker A motifs (GQSLSGKS, residues 96-103) within the two βγ-crystallin domains of the βγ-CAT α-subunit, which are responsible for the formation of nucleotide binding site and can be commonly found in a number of ATP- and GTP-binding and hydrolyzing proteins (Walker et al, 1982). βγ-CAT showed potential NTP-binding and weak GTPase/ATPase activities *in vitro*. It suggests that βγ-CAT may be a novel GTPase/ATPase or ATP- and GTP-binding and hydrolyzing protein. We suppose that it maybe acts as a new regulator of cell function (He et al, 2008b).

In conclusion, it is the first time to report that βγ-CAT could induce sustained contraction of rabbit aortic rings in dosage and endothelium dependent manners in this paper. Our findings will certainly provide new clues to understand the functions and physiological role of non-lens βγ-crystallins and trefoil factor. In addition, we anticipate that our related research works may be a new starting point for the understanding of functional positions of non-lens βγ-crystallins and trefoil factors in cell proliferation, differentiation and apoptosis signaling net, providing novel view in the study and understanding of human diseases, like tissue regeneration, cancer and heart failure.

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(上接第 476 页)

图 1　欧夜鹰巢、卵、雏鸟和成鸟（2008 年 6—8 月，马鸣野外拍摄）

Fig. 1　Eggs and chicks of Eurasian Nightjar in field from June to August 2008, photos by MA Ming

(下转第 510 页)