The effects of Cyclic guanosine 3', 5'-monophosphate analog on protein accumulation in adult rat cardiomyocytes in vitro

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ABSTRACT

Cyclic guanosine 3', 5'-monophosphate (cGMP) has recently emerged as an endogenous regulator for controlling or reversing cardiac hypertrophy. Increased protein accumulation is a key feature of cardiac hypertrophy; thus, our study investigates the effects of a cGMP analog on protein accumulation in primary culture of adult rat cardiomyocytes and dissects out the mechanisms involved. We confirmed that a cGMP analog, 8-bromo-cGMP, inhibits phenylephrine (PE)-increased accumulation of newly synthesized proteins in cultured adult rat ventricular cardiomyocytes. Firstly, we have obtained data showing that 8-bromo-cGMP does not inhibit phosphorylation of S6K1 by PE during short time treatment (10 min to 2 h), but blocks phosphorylation of S6K1 by PE at 6 h; moreover this blocking effect is completely abolished by phosphatase inhibitor Tautomycin. Then, we have demonstrated that PE and cGMP induce sustained and transient increased phosphorylation of ERK, respectively. Moreover, cGMP inhibits PE-induced phosphorylation of ERK during long term treatment (3 and 6h). We have also shown that 8-bromo-cGMP inhibits ROS generation induced by PE. Other effects of PE that could be related to hypertrophy (i.e. increased concentration of upstream binding factor mRNA and decreased concentration of the mRNAs of Atrogin and muscle specific RING finger) were not abolished by 8-bromo-cGMP. We conclude that cGMP analog blocks protein accumulation by inhibiting the sustained phosphorylation of S6K1 via the activation of phosphatases.

RÉSUMÉ

Il a été montré récemment que la guanosine 3',5'-monophosphate cyclique (GMPc) est un régulateur endogène qui contrôle ou abolit l'hypertrophie cardiaque. L'accumulation de protéines est une caractéristique clé de l'hypertrophie cardiaque. Aussi, nous nous sommes intéressés aux effets d'un analogue du GMPc sur l'accumulation de protéines dans des cultures primaires de cardiomyocytes de rats adultes, et nous avons caractérisé les mécanismes moléculaires mis en jeu. Nous avons confirmé que le 8-bromo-GMPc, un analogue du GMPc, inhibe la synthèse protéique médiée par la phényléphrine (PE) dans des cultures primaires de cardiomyocytes de ventricules de rats. Premièrement, nous avons montré que le 8-bromo-GMPc n'inhibe pas la phosphorylation de S6K1 après un court traitement à la PE (10 à 120 min), mais inhibe cette même phosphorylation après 6h de traitement à la PE. De plus, cet effet est complètement bloqué par la tautomycine, un inhibiteur de phosphatases. Ensuite, nous avons démontré que la PE et le cGMP induisent une augmentation respectivement prolongée et transitoire de la phosphorylation de ERK. Le cGMP inhibe la phosphorylation de ERK induite par la PE pendant un traitement prolongé (3 à 6 h). Nous avons aussi montré que le 8-bromo-GMPc inhibe la production de radicaux libres induite par la PE. D'autres effets de la PE qui pourraient être reliés à l'hypertrophie (comme l'augmentation de l'expression des ARNm de facteurs de liaison en amont et la diminution de l'expression des ARNm de l'atrogine et des « RING finger » spécifiques du muscle) n'ont pas été inhibés par le cGMP. Nous concluons que le 8-bromo-GMPc bloque l'accumulation de protéines en inhibant la phosphorylation prolongée de S6K1 via l'activation de phosphatases.

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SECTION 1: INTRODUCTION

Hypertrophic ventricular remodeling is a response of the heart to numerous forms of cardiac stresses, including increases in workload, loss of contractile mass from prior infarction, and/or neuroendocrine activation[1]. Whereas it may initially represent compensatory response, it often precipitates (when present chronically over extended periods of time) the development of more serious complications, such as dilated cardiomyopathy, overt heart failure, arrhythmia, fibrotic disease, and sudden death[2]. In such conditions, cardiac hypertrophy is usually recognized as a maladaptive response, which makes it necessary to identify the underlying molecular events. This in turn may lead to the development of strategies to prevent or reverse the hypertrophic phenotype, and thus enable us to prevent development of the maladaptive complications of ventricular hypertrophy.

Cardiac hypertrophy is characterized by an enlargement of the heart associated with an increase in cardiomyocyte cell volume, enhanced protein accumulation, a higher degree of sarcomeric organization and re-expression of certain fetal genes[3]. Increased protein accumulation which represents the balance between protein synthesis and degradation, is a necessary mediator of cardiac hypertrophic growth. Under hypertrophic stimuli, cardiomyocytes increase both protein synthesis and degradation, but, the increase in the rate of protein synthesis is higher than that of protein degradation, leading to a net increase in protein accumulation[4].

Although we have already developed effective drugs to reduce hypertrophy, cardiovascular disease still remain in the leading cause of the death in Western countries[5].

Cyclic guanosine 3', 5'-monophosphate (cGMP), synthesized in response to diverse signals, has recently emerged as an endogenous negative regulator for cardiac hypertrophy. This signaling pathway might provide the basis of novel strategies to control or reverse hypertrophy. However, the mechanisms by which cGMP regulate protein accumulation in cardiomyocytes have not yet been explored in detail.

Characteristics of cardiac hypertrophy

In mammals, it is generally believed that adult cardiomyocytes are terminally differentiated cells that have withdrawn from the cell cycle during the perinatal period[6,7]. In response to sustained increases in blood pressure and/or hormonal challenges, the heart triggers a hypertrophic response to counterbalance the increase in wall stress in order to increase pump function and adapt to new work conditions[8]. Cardiac hypertrophy is characterized by an enlargement of the heart associated with an increase in cardiomyocyte cell volume, enhanced protein accumulation, a higher degree of sarcomeric organization and re-expression of certain fetal genes[3]. Cardiac hypertrophy can be classified as either physiological or pathological hypertrophy. Exercise training can induce so-called physiological hypertrophy, where cardiac function is preserved. Diverse pathological stimuli (including hypertension, valve diseases, myocardial infarction and genetic mutations) can trigger pathological hypertrophy, which is associated with cardiac dysfunction and increased mortality. Based on the change in shape, cardiac hypertrophy can be distinguished as eccentric and concentric hypertrophy. Eccentric hypertrophy, which may develop as a consequence of volume overload or after infraction, is characterized by addition of sarcomeres in series and longitudinal cell growth. Concentric hypertrophy, which may develop as a consequence of pressure overload, is characterized by addition of sarcomeres in parallel and lateral cell growth[9]. At the cellular level, cardiac hypertrophy is characterized by an increase in cardiomyocyte size, increased protein accumulation, and heightened organization of the sarcomere structure. At the molecular level, these changes are accompanied by the re-induction of fetal gene program, including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and

genes for fetal isoforms of contractile proteins, such as sketetal α-actin and β-myosin heavy chain (MHC), and by a down-regulation of genes normally expressed in adult such as α-MHC and sarcoplasmic reticulum Ca^{2+}-ATPase[10,11].

The molecular pathways of cardiac hypertrophy

Numerous cardiomyocyte-autonomous and endocrine/paracrine pathways have been implicated in the molecular response to the development of cardiac hypertrophy as detailed in the following paragraphs.

Calcineurin- NFAT signaling

Increased intracellular Ca^{2+} activates calcineurin, a Ca^{2+}-dependent serine-threonine protein-phosphatase, which subsequently dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factor, enabling it to translocate to the nucleus and activate prohypertrophic gene expression [12]. It has been shown that the calcineurin-NFAT pathway is both necessary and sufficient for mediating cardiac hypertrophy[13]. Constitutive activation of calcineurin or overexpression of constitutively activated NFAT3 is sufficient to induce cardiac hypertrophy[13]. Several molecules that act as specific endogenous inhibitors of calcineurin, including AKAP79, Cabin/Cain and myocyte-enriched calcineurin-interacting protein (MCIP), abolish the hypertrophic growth induced by multiple hypertrophic stimuli [14,15]. Overexpression of MCIP, which binds calcineurin and inhibits its activity, not only inhibits cardiac hypertrophy induced by various different stimuli, it also does not result in deterioration of systolic function in spite of the lack of compensatory hypertrophy during 3 months of observation[14]. These

findings support the notion that calcineurin-NFAT signaling plays a critical role in pathological response and is not required for compensatory response. Overexpression of MCIP1 in transgenic mouse heart also attenuates exercise-induced cardiac hypertrophy, which suggests that calcineurin is also involved in physiological hypertrophy[14]. Although the involvement of calcineurin-NFAT signaling in pathological hypertrophy is well-established, the therapeutic potential of inhibiting this pathway is limited by its involvement in physiological hypertrophy, by its protective role against apoptosis[16], and by the lack of tissue specificity of pharmacologic inhibitors as well as their potential toxic effects.

G-protein-Coupled Receptor (GPCR)

Myocardial GPCRs, including adrenergic, endothelin-1 (ET-1), and angiotensin II receptors play an important role in the development of cardiac hypertrophy as functionally redundant transducer. GPCRs are seven-transmembrane-spanning receptors coupled to the heterotrimeric GTP binding proteins G_s, G_q/G_{11} and G_i, all of which consist of the G_α and the $G_{\beta\gamma}$ subunits[17]. Angiotensin II (Ang II), ET-1 and α-adrenergic receptor agonists bind to G_q/G_{11}–coupled GPCRs to activate phospholipase C β (PLCβ). The latter hydrolyses phosphatidylinositol 4, 5-triphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP_3). Pressure overload also activates similar G_q/G_{11}–coupled GPCRs by mechanisms that have not been well-elucidated yet. IP_3 binds to receptors in the sarcoplasmic reticulum (SR), releasing Ca^{2+} which can activate calcineurin-NFAT or calmodulin-dependent kinase (CaMK)-histone deacetylase (HDAC) signaling. DAG activates protein kinase C (PKC) isoforms, leading to the release of Ca^{2+}

from SR, with or without the involvement of IP_3. Multiple studies have implicated PKC isoforms in the development of cardiac hypertrophy[18,19]. It has also been shown that PKCs regulate mammalian target of rapamycin (m-TOR) by activating mitogen-activated-protein kinase (MAPK)[20,21]. Activation of the βγ subunits of G_q/G_{11}–coupled GPCRs activates the phosphoinositide-3 kinase (PI3K) isoform p110γ, leading to the activation of AKT-mTOR signaling[20]. p110 γ is required for stress-induced hypertrophy, but not for physiological hypertrophy[22]. Cardiac-specific transgenic overexpression, dominant inhibition and gene ablation of GPCRs have proven that GPCR signaling pathway is both necessary and sufficient to induce cardiac hypertrophy in multiple pathologically hypertrophic stimuli[23-25]. Cardiac-specific overexpression of $G_{\alpha q}$ induces cardiac hypertrophy and cardiac dysfunction[23,26]; while cardiac-specific ablation of GPCR did not result in cardiac hypertrophy in response to pressure overload[25]. Furthermore, the absence of impaired cardiac function when $G_{q/11}$ is inhibited indicates that GPCR signaling is not required for functional compensation. This supports the notion that cardiac hypertrophy is not a prerequisite adaptive phenomenon, at least in the experimental animal models during the observation period. β-adrenergic receptor agonists bind to β_1-receptors, which couple to Gs, leading to activation of adenylate cyclase (AC). AC generates cAMP to activate protein kinase A (PKA), eventually resulting in positively chronotropic, inotropic effect on heart. β_2-adrenergic receptors couple to Gs and Gi, and Gi inhibits AC to oppose Gs-dependent signaling[2].

PI3K/Akt/mTOR signaling

Insulin-like growth factor-1(IGF-1), insulin and other growth factors bind to tyrosine kinase receptors and trigger translocation of the PI3K isoform p110α to the cell membrane. p110α phosphorylates phosphatidylinositols (PIP_3) to recruit both Akt and PDK1 to the membrane, allowing PDK1 to activate Akt. Activated Akt activates mTOR, a central regulator of protein synthesis, to increase protein synthesis. On the other hand, Akt also phosphorylates and inhibits glycogen synthesis kinase-3 (GSK-3), which inhibits protein translation and transcription factors[27,28]. It has been shown that the PI3K/Akt signaling pathway transduces physiological cardiac hypertrophy[29]. However, the PI3K isoform p110γ is also activated when the βγ subunits of activated $Gq/_{11}$ are recruited, and then activates Akt and mTOR; the latter being required in pathological hypertrophy[22]. Both physiologically and pathologically hypertrophic stimuli can activate PI3K/Akt pathway, but they may differ in their ability to activate p110α or p110γ, respectively. However, it is believed that the major difference between them is the recruitment of PLCβ/Ca^{2+} singaling by Gq[20].

The MAPK cascades

The MAPK pathways provide an important link between external stimuli and the nucleus via phosphorylation and regulation of multiple transcription factors. The MAPKs are final components of a cascade of 3 consecutive kinases: MAPKKKs activate MAPKKs, and then MAPKKs activate MAPKs. There are three subfamilies of MAPKs: extracellular response kinase (ERK), C-Jun N-terminal kinase (JNK) and P38 MAPK[30]. GPCRs, tyrosine kinases and stress all can trigger MAPK signaling cascades[31,32]. some

investigators have suggested that ERK1/2-dependent signaling is sufficient to induce hypertrophy[33]. Overexpression of MAPK phosphatase-1, which inhibits all three MAPKs signaling, blocks both pressure overload-induced and agonist-induced cardiac hypertrophy, thus confirming the critical role of MAPK pathway in hypertrophy[34].

Small GTP-Binding Proteins

There are five families of small GTP-binding proteins: Rho, Ras, ARFs, Rab and Ran, and several small GTPases are involved in hypertrophy. Overexpression of Ras in transgenic mouse heart and neonatal rat cardiomyocyte increases myocardial mass and hypertrophic gene expression[35,36]. The Rho subfamily, which includes RhoA, Rac1 and cdc42, also appears to play a relevant role in hypertrophy[37]. Overexpression of RhoA in transgenic mouse heart is not sufficient to induce cardiac hypertrophy, but leads to conduction abnormalities and heart failure[38]. Inhibition of RhoA by dominant-negative RhoA mutants or inhibition of Rho-associated kinase (ROCK) prevents cardiac hypertrophy *in vivo*[39]. RhoA activates ROCK, which activates myosin light chain kinase (MLCK), mediating sacomeric reorganization[40]. RhoA also can activate serum response factor (SRF), which is a transcription factor involved in hypertrophic signaling and the regulation of muscle-specific genes (such as ANF and α-skeletal actin) by changing actin dynamics[41,42]. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), inhibit hypertrophy, as well as decrease activity of Rac1 and RhoA, indicating that it may exert antihypertrophy by suppression of small G-protein signaling[43].

Na^+/H^+ Exchanger (NHE)

NHE is a ubiquitously expressed membrane glycoprotein which regulates intracellular pH and concentration of Na^+. The activity of cardiac NHE is increased in several animal models of cardiac hypertrophy[2]. Increased NHE activity leads to increased intracellular Na^+ level, which upregulates Na^+/Ca^{2+} exchanger, mediating elevated intracellular Ca^{2+} levels and consequent signaling cascades[44]. Cariporide, an NHE inhibitor, has been shown to prevent hypertrophy *in vivo*[44,45].

Histone Acetylation /Deacetylation

Histone acetyltransferases (HATs) acetylate histones to relax chromatin structure and activate gene transcription; while HDACs decetylate histones to compact chromatin and repress gene transcription[46,47]. HATs and HDACs also serve as intracellular targets for signaling cascades from the cell surface and thereby couple extracellular signals to the genomes. HATs and HDACs play a critical role in modulating the activities of transcription factors such as GATA4, NFAT, SRF, myocyte enhancer factor (MEF), and cAMP responsive element-binding protein (CREB), which are implicated in hypertrophic gene expression[48-51]. HATs, p300 and CREB binding protein (CBP) are enhanced when cardiac hypertrophy is induced by stimulation of the MAPK pathway by the α-adrenergic receptor agonist phenylephrine (PE) [48]. Dominant negative expression of p300 blocks PE-induced cardiac hypertrophy, whereas overexpression of p300 results in hypertrophy[49]. CaMK, either directly or by phosphorylating HDAC kinase, phosphorylates HDACs to induce their export out of the nucleus, and thus release depression on transcriptional activity[50,52]. Class II HDACs are involved in hypertrophic growth through interaction

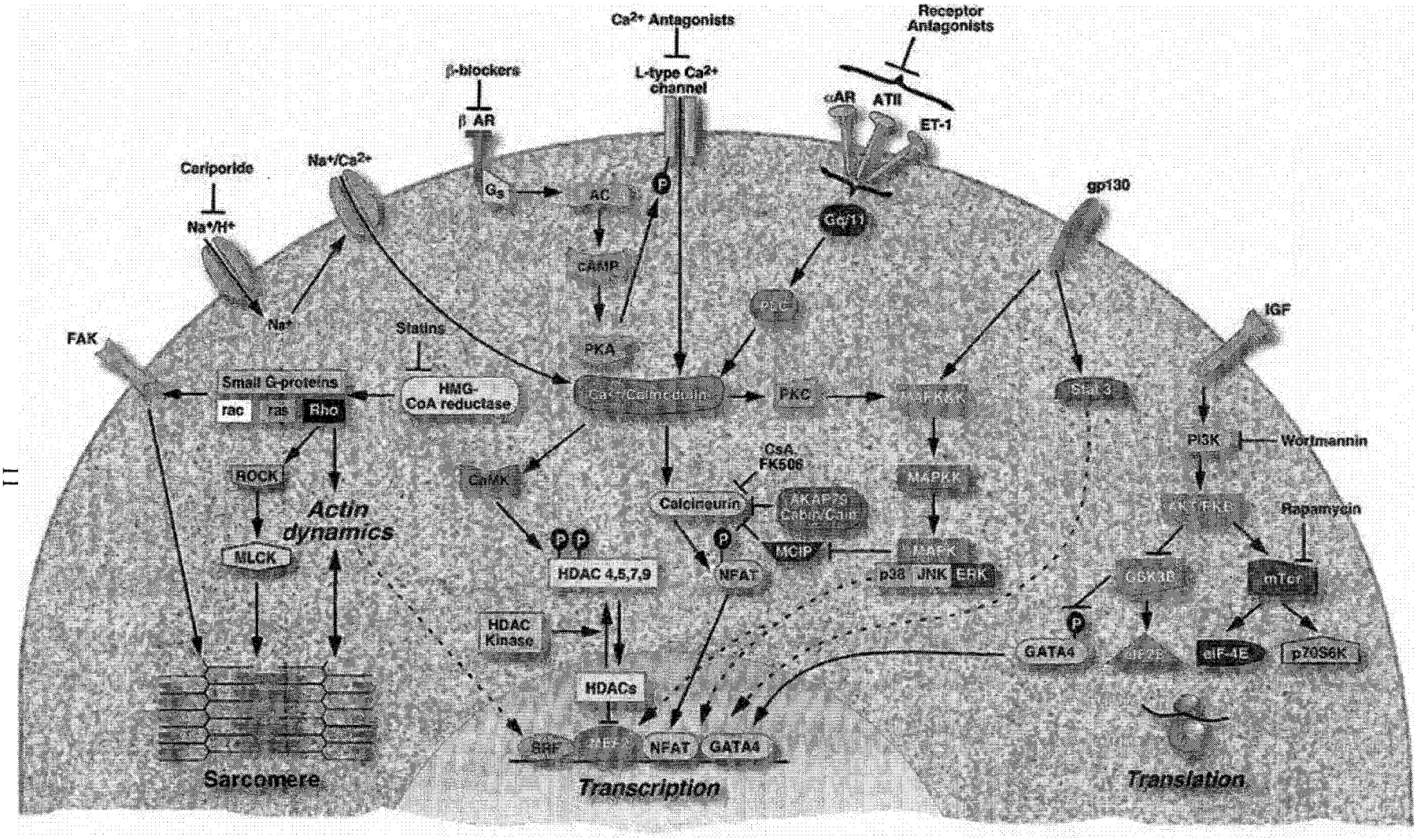
with transcription factors such as MEF-2[50,51]. However, in comparison with class II HDACs, class I HDACs seems to act in an opposite way. Inhibition of class I HDACs almost completely blocks the cardiac hypertrophy induced by pressure overload and Ang II [53,54]. Recruitment of either class I or class II HDACs may thus regulate the balance between prohypertrophic and antihypertrophic transcriptional process.

Reactive Oxygen Species (ROS)

ROS include radicals such as superoxide and nonradicals such as hydrogen peroxide, and they participate in both physiological and pathological process. It has been demonstrated that ROS are involved in cardiac hypertrophy. Different hypertrophic stimuli such as α-adrenergic receptor agonists, Ang II, ET-1, mechanical stretch and pressure overload increase ROS production *in vitro* or *in vivo*, and antioxidant may, in addition to reducing the abundance of ROS, also inhibit cardiac hypertrophy [55-59]. It also has been suggested that ROS are involved in cardiac hypertrophy by activating multiple signaling pathways such as MAPK, ERK1/2, P38, JNK, PKC, RhoA family and NHE[55,60-62].

A summary of the various molecular events involved in cardiac hypertrophy is shown in Figure1.

Figure 1. The various molecular events involved in cardiac hypertrophy



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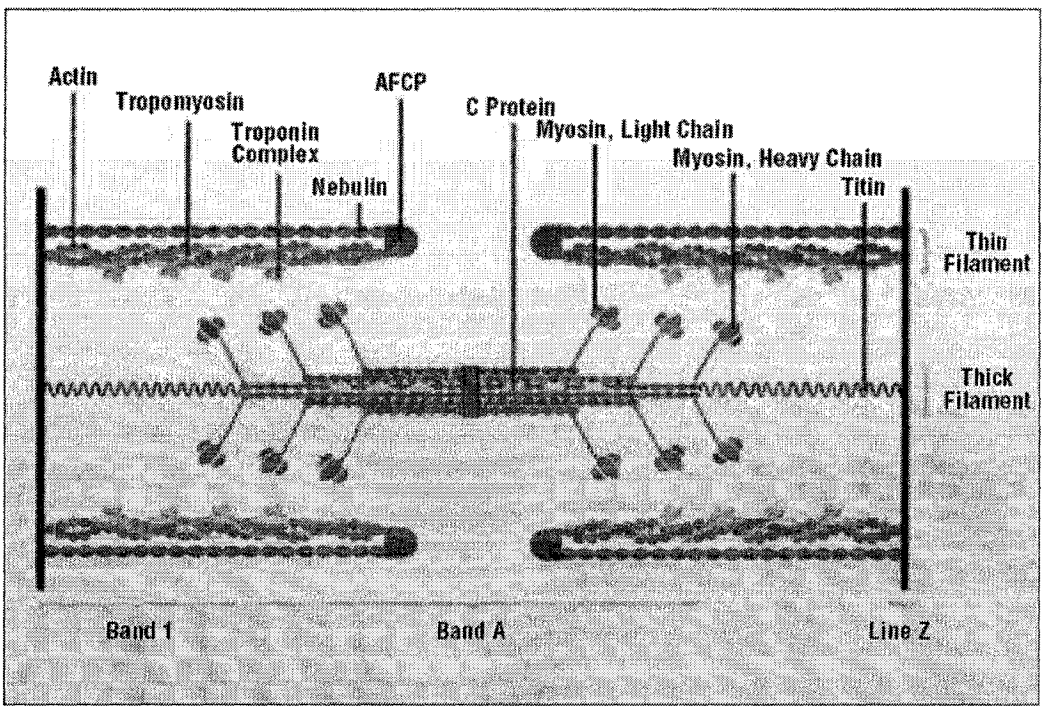
Protein accumulation in cardiac hypertrophy

Protein accumulation reflects both protein synthesis as well as protein degradation. During cardiac hypertrophy, both protein synthesis and degradation are accelerated, but protein synthesis rate is higher than protein degradation, leading to a net increase in protein accumulation[4]. Deposition of synthesized protein leads to the growth of cardiomyocytes, including the addition of sarcomeres (sarcomerogenesis); (Figure 2. show the structure of sarcomere). Both inhibition of protein synthesis and activation of protein degradation can serve as an effective strategy to prevent or reverse cardiac hypertrophy, so it is necessary to understand the pathways regulating protein synthesis and degradation.

Figure 2. The structure of sarcomere



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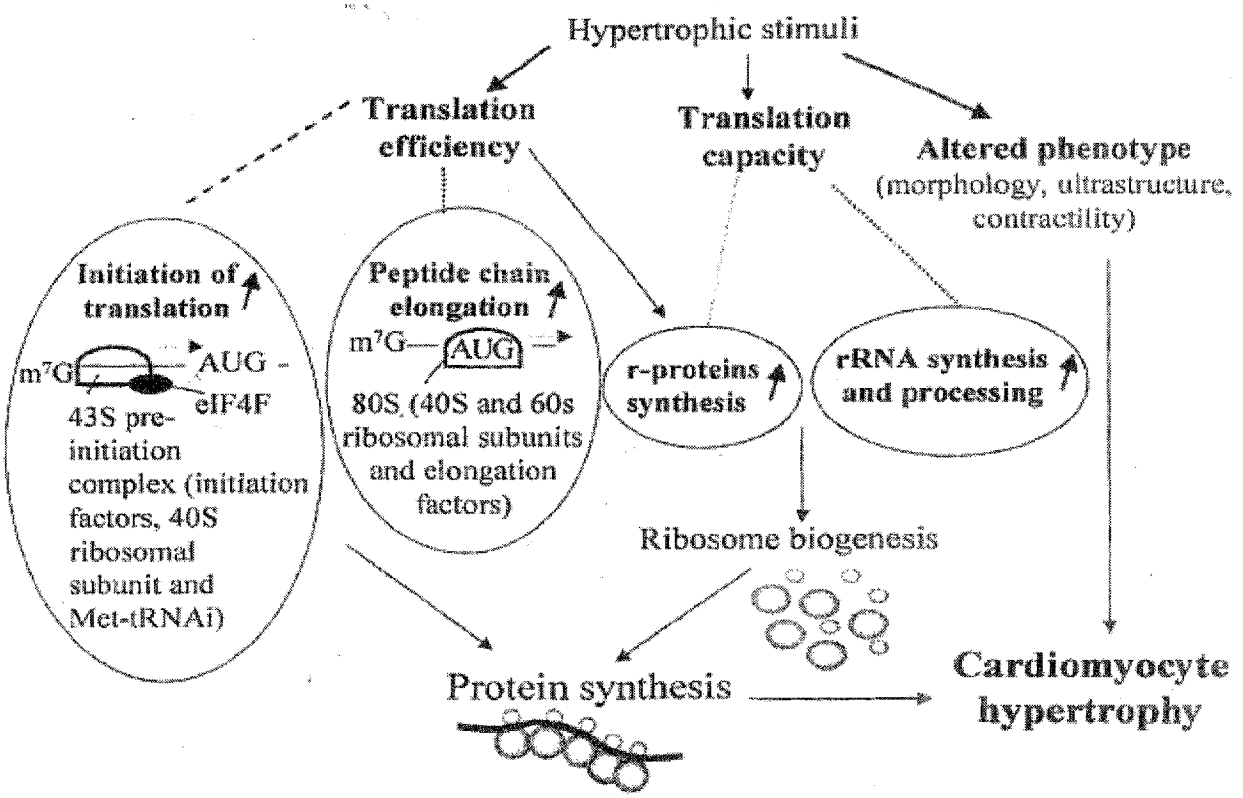
Regulation of protein synthesis

Protein synthesis can be regulated at the level of either transcription or translation, as well as by post-transcriptional modifications[4]. The abundance of mRNAs coding for specific structural proteins (such as α-, β-myosin heavy chain (MHC) and α-actin) has been shown to be regulated in the course of hypertrophy (by either transcriptional or post-transcriptional events) [63,64], but these events cannot account for the increase in global protein synthesis associated with cardiac hypertrophy. Likewise, it has been suggested that the availability of mRNA is not generally a limiting factor for protein synthesis, which means that translation is the critical step to regulate protein synthesis[4]. Increased protein translation can be due to increased translation efficiency and/or capacity. Translation efficiency is the rate of translation of mRNA pool and translation capacity is the amount of the protein synthesis machinery. Studies on expression of cardiac-specific MHC gene during pressure overload have shown that increased MHC protein expression results in short-term experiment (4-6h) from an increase in the number of active polysomes translatating MHC mRNA, whereas during long-term pressure overload, it is due to increases in the abundance of both ribosomes and active polysomes [65,66]. These studies suggest that the hypertrophic growth response is biphasic, where translation efficiency is increased during the acute phase, and then followed by an increase in translation capacity during sustained growth demand (figure 3).

Fiugre 3. The biphasic changes of protein accumulation due to hypertrophic stimuli.



The acute phase is due to increased protein translation efficiency; followed by both rises in translation efficiency and capacity due to long term hypertrophic stimuli. (Hannan R.D. 2003)

Regulation of translation efficiency

mRNA translation consists of the processes of initiation, elongation and termination, with the initiation process being a key point in controlling translation in mammals[67]. The process of translation initiation starts from initiator methionyl-tRNA (Met-tRNAi) binding to different eukaryotic initiation factors (eIFs) and 40s ribosomal subunit to form 43s preinitiation complex. The assembly of 43s and eIF4F complex (eIF4A, eIF4E and eIF4G) recruits 5'ends mRNA harbouring a 7-methylguanosine (m^7G) cap (5'-CAP mRNA), and then scans along 5'end mRNA until initiation codon is paired to Met-

tRNAi. Following the release of the 40s subunits, the 60s subunit joins to form 80s ribosome to proceed into elongation[68]. The eukaryotic initiation factor 2B (eIF2B) and 4E (eIF4E) are key factors that regulate the initiation of translation. eIF2 transfers Met-tRNAi to the ribosome and GTP-bound eIF2 is hydrolyzed to GDP-bound eIF2 during this process. Guanine nucleotide-exchange factor eIF2B catalyzes GDP-bound eIF2 to active GTP-bound form. Hypertrophic stimuli cause inactivation of GSK-3 and bring about the dephosphorylation and activation of eIF2B to increase translation initiation[69,70]. eIF4E is required for formation of eIF4F complex, and eIF4E binding proteins (4E-BPs, 4E-BP-1 is the most widely studied) interact with eIF4E to prevent it from binding to eIF4G to form the eIF4E complex. Hypertrophic stimuli induce activation of mTOR, which then phosphorylates 4E-BPs, leading to their dissociation from eIF4E. Released eIF4E then joins the eIF4F complex to increase initiation of translation[71]. Furthermore, activated mTOR phosphorylates S6 kinases (S6Ks), which directly dephosphorylate eEF2 kinase to activate eEF2, mediating increased peptide-chain elongation[72,73]. Moreover, it has been recently shown that eukaryotic translation initiation factor 4B (eIF4B) is phosphorylated by S6K1 and then facilitates eIF4A to recruit to initiation complex[74,75].

Regulation of translation capacity

Translation capacity is determined by ribosomal biogenesis, which consists of r-RNA and r-proteins synthesis. Compelling evidences have shown that the rate of 45S ribosomal gene (rDNA) synthesis by RNA polymerase I is the key step for ribosomal biogenesis[76,77]. During cardiac hypertrophic growth, increased protein synthesis is accompanied by accelerated transcription of 45S ribosomal gene *in vivo and in vitro*[78,79]. Furthermore, an increase in rRNA synthesis is required for accelerated growth of cardiomyocytes[78].

Increased rDNA transcription rate can account for chromatin remodeling, amounts and activities of RNA polymerase I and rDNA transcription factors. Upstream binding factor (UBF) has been strongly implicated in the regulation of rDNA transcription. In mammalian cells, at least two DNA-binding proteins, UBF and the core rDNA-specific transcription factor selectivity factor-1 (SL-1), as well as RNA polymerases I are required for efficient rDNA transcription *in vitro*[79]. Purified mammalian UBF contains two proteins of molecular sizes 97 and 94 kDa, referred to UBF1 and UBF2, respectively. These two proteins result from alternative splicing of the same primary transcript[78]. Both of UBF1 and UBF2 can bind to DNA to form homo- and heterodimers by interacting with the rDNA promoter via DNA binding domain (HMG boxes)[80]. However, it has been shown that UBF1 is four times more efficient at promoting rDNA transcription than UBF2[81]. The activity of UBF is regulated at the transcriptional and/or post-translational levels. the latter including phosphorylation, acetylation or interaction with other proteins[81-83]. UBF is a phosphoprotein, and phosphorylated UBF is more active than dephosphorylated UBF[84]. Hypertrophic stimuli such as α_1-adrenergic receptor agonists and contractile activity increase UBF expression level[85,86], whereas endothelin-1 increases UBF phosphorylation[87]. Overexpression of UBF alone in non-hypertrophied neonatal cardiomyocytes mediates an increase in rDNA transcripition[88]. Furthermore, inhibition of UBF by UBF antisense RNA in cardiomyocytes results in a significant reduction in rDNA transcription, protein accumulation and a decrease of cell size, but does not alter the fetal gene program which is regulated by RNA polymerase II[78]. All together, these studies suggest UBF is sufficient and necessary for increased rDNA synthesis in hypertrophic growth.

r-protein synthesis

r-proteins is actively regulated during hypertrophy and produced in equimolar amouts as the ribosomal subunits to form functional ribosomes [89,90]. 5'-terminal oligopyrimidine tract mRNAs (5'-TOP mRNA) encode r-proteins and several eIF translation factors. Hypertrophic stimuli increase the translation rate of 5'-TOP mRNA[91]. Ribosomal S6 protein (rpS6) had first been considered as the only S6K substrate and the positive regulator for protein synthesis for many years. Different hypertrophic stimuli phosphorylate and activate S6K, which subsequently phosphorylates and activates S6. There are two forms of S6K, S6K1 (p70/85 kinase1) and S6K2 (p54/56 kinase2), which are highly homologous, but differ in N and C-terminal regions. S6K lies downstream of PI3/Akt/mTOR, but is also activated by MAPK/ERK and PKC pathways[92,93]. Different hypertrophic stimuli such as GPCR agonists, insulin and pressure overload can phosphorylate S6K. Inhibition of S6K by rapamycin, which blocks the activity of mTOR, leads to suppression of 5'-TOP mRNA translation under hypertrophic stimuli[94,95]. Overexpression of S6K1, but not S6K2 (the dominant S6K in the heart) induces cardiac hypertrophy to a modest degree. The deletion of S6K1, but not S6K2, results in animals where the heart is 20% smaller than in wild-type counterparts, but does not attenuate exercise-, pressure overload-, IGF-1/PI3K-dependent cardiac hypertrophy [96]. This study suggests that S6Ks may not be critical for hypertrophic growth, but the possibility remains that a compensatory pathway in S6K-knock out mice may mask the role of S6Ks. There has been evidence suggesting which support that phosphorylation of rpS6 induced by S6K increases the affinity of ribosomes for 5'-TOP mRNAs, and thus facilitates the translation of this class of mRNA. While this has been widely accepted, and also

considered as the mechanism by which S6K increases protein synthesis, other studies in cultured cells have questioned this conclusion. Montine K.S. et al. have reported that phosphorylation of rpS6 does not increase protein synthesis in Ehrlich cells[97]. In S6-null cells, the total amount of ribosome and the growth of hepatocytes were unchanged[98]. The discrepancy between these studies has been recently resolved by the finding that rpS6 $^{p-/-}$ mice do not affect the translation of 5'-TOP mRNA, and exit a significantly higher rate of global protein synthesis rate in mouse embryo fibroblasts compared to wide type. This study also indicates that translation of 5'-TOP mRNA is controlled by S6K/rpS6 independent pathway. Because other S6K substrates have emerged recently (such as eIF4B, eEF2K, mTOR), it is that other substrates are responsible for the positive effect of activated S6K1 on protein synthesis[72-75,94,95]. Moreover, S6K1 also directly activates rDNA transcription in cardiomyocytes[99]. It is reasonable to hypothesize that increased activity of S6K might act as fine tuning regulator for protein synthesis. S6K exerts reciprocal effects which downregulate protein synthesis by phosphorylation of rpS6 and upregulate protein synthesis by activation of other substrats[100]. (figure 4. show the signaling pathway involved in protein synthesis)

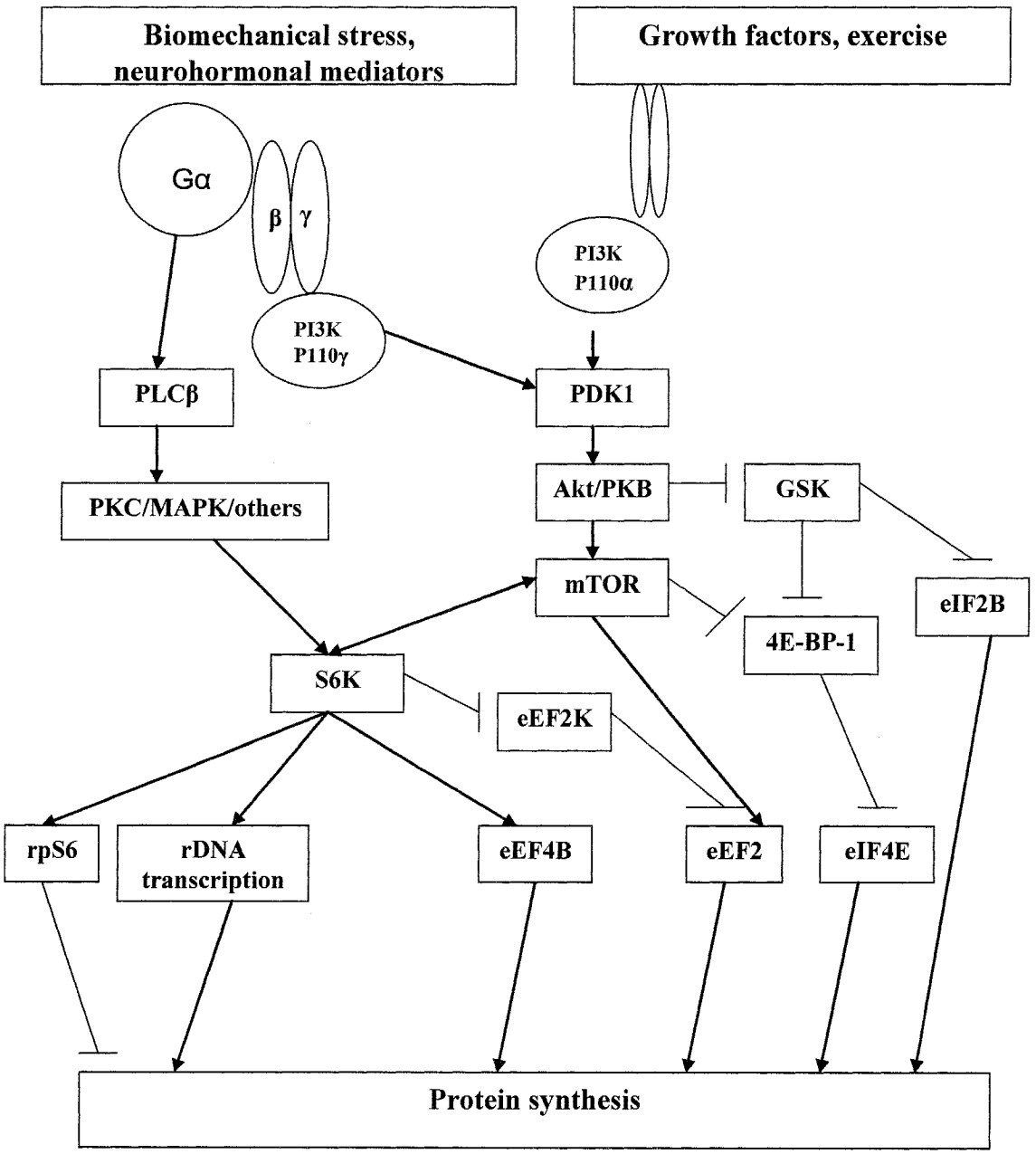


Figure 4. The signaling pathways involved in protein synthesis in cardiac hypertrophy

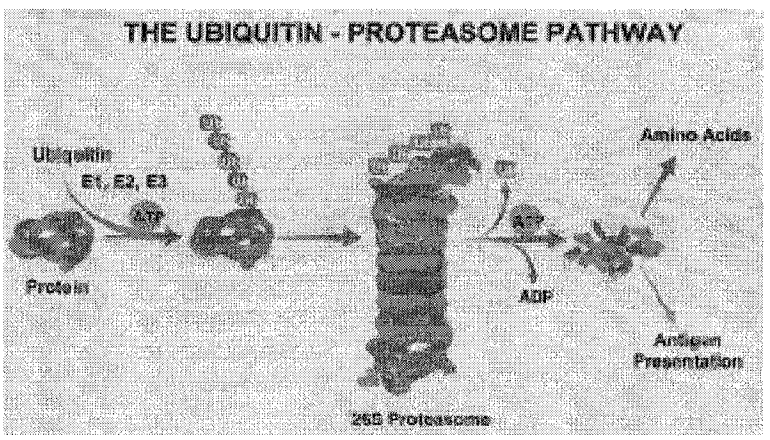
Regulation of Protein Degradation

Activation of protein degradation may be more efficient than inhibition of protein synthesis to reverse hypertrophy, because the prohypertrophic signaling network is very redundant, and activation of protein degradation may be effective regardless of the nature of the pro-hypertrophic stimulus. So it is important to dissect the molecular events involved in protein degradation to apply this novel strategy.

In striated muscle, there are three major proteolytic systems for protein degradation: (a) the lysosomal protease system; (b) the cytosolic protease system; and (c) the ubiquitin proteasome system (UPS)[101]. UPS has recently received more attention in the regulation of protein accumulation in cardiac hypertrophy and holds promise for new therapies. Lysosomes are cell organelles containing the acid proteases called cathepsins. Lysosomal proteases are responsible for degradation of extracellular and membrane proteins (e.g. membrane receptors), but are not important for myofibrillar proteolysis[102]. Cytosolic proteases include the calcium-dependent calpains, which are involved in the early process of proteolysis and the disassembly of myofibrillar proteins. Desintegrated myofibrillar proteins are then made available to the UPS for further degradation. This process occurs in the course of ischemia reperfusion-induced injury and is activated during atrophic remodeling[103,104]. UPS is the major proteolytic system in striated muscle cells, as it degrades about 80% of the intracellular proteins in such cells via an ATP- dependent pathway, and is the most important pathway for the degradation of myofibillar proteins, i.e. the most abundant muscle proteins[105]. There are three steps in the process of ubiquitin-proteasome degradation: first, ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent reaction. The activated ubiquitin is then transferred to

ubiquitin-conjugating enzyme (E2). Finally, the ubiquitin ligases (E3) catalyze C-terminal carboxy group of ubiquitin link to ϵ-amino group of amino acid of the lysine residue of the protein substrate. Repeated cycles of ubiquitin conjugation result in formation of a polyubiquitin chain on the target protein, which is then recognized by the 19S subunits of proteasome. Subsequently, the ubiquinated protein is degraded into peptides containing less than 20 amino acids in the 20S core proteasome. These peptides are then completely hydrolyzed by cytosolic peptidases[106]. UPS functions as quality control system, as it acts on the degradation of denatured and misfolded proteins; and regulates the steady state level of biologically regulatory proteins[107].

Figure 5. The ubiquitin-proteasome pathway



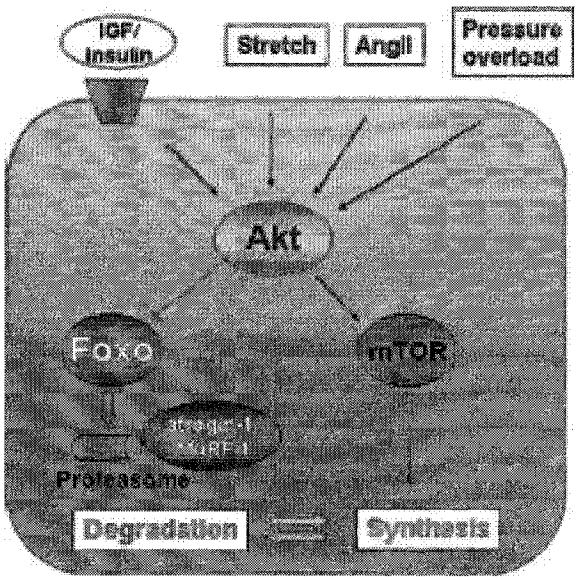
ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme E1, which then transfers the highly reactive ubiquitin to ubiquitin-conjugating enzyme E2. ubiquitin ligase E3 transfers the ubiquitin from the E2 onto ϵ-amino group on a lysine on the protein substrate. Cycles of ubiquitin by the same E3 forms polyubiquitin chain on the protein for 26s proteasome degradation. (Epstein F. H. 1996)

There are several types of E2s and about 1,000s of E3s. Each pairing of E2/E3 enzymes recognizes only a limited subset of target proteins and keeps the specificity of UPS. Each

E3 recognizes a specific amino acid at the N-terminus of the target proteins. E3s can also target some proteins when they are altered by post-translational modifications such as phosphorylation, acetylation or methylation[108]. E3s can be classified into three families: those containing homologous to E6-AP carboxy terminus (HECT) domains, those containing RING fingers and the Skp-Cullin-F-box (SCF) family[109]. The function of E3s in protein degradation came from the muscle atrophy literature. Recently two specific E3s, atrogin-1 (SCF family) and muscle specific RING finger-1 (MuRF-1), have received much attention in the context of cardiac hypertrophy. In parallel with increased muscle proteolysis, muscle ubiquitin mRNA, proteasome subunit mRNAs and polyubiquitinated proteins are all increased in multiple atrophic conditions such as starvation, fasting and denervation[110,111]. It has been shown that atrogin-1 interacts with calcineurin and represses calcineurin by targeting it for ubiquitin-mediated proteolysis. Overexpression of atrogin-1 decreases calcineurin A level, inhibits NFAT translocation, attenuates agonist-induced calcineurin activity and blunts cardiac hypertrophy after pressure overload[112]. MuRF-1 interacts with receptor for activated protein kinases C (RACK1), blocks PKCε translocation to focal adhesions and inhibits focal adhesion formation and downstream effectors ERK1/2 during PE and PMA stimulation. MuRF-1 inhibits phenylephrine (PE), PMA-induced increase in cell size and sarcomere reorganization[113]. It has also been shown that MuRF-1 interacts with the titin protein to disrupt the sarcomere assembly, and it targets troponin I (TnI) for preoteasome-dependent degradation[114]. Recent studies have shown that the Forkhead families of transcription factor FOXO proteins play a central role in regulating atrogin-1 and MuRF-1. FOXOs are downstream of Akt, are negatively regulated by PI3K/Akt signaling pathway[115-117], and activate the transcription of Atrogin-1 and MurF-1. Multiple hypertrophic stimuli such as IGF, insulin, pressure overload and

AngII activate the PI3K/Akt pathway to phosphorylate and inhibit FOXOs. Overexpression of FOXO3a gene prevents hypertrophy *in vitro* and *in vivo*, and constitutively active FOXO3a transduction increases the transcription of atrogin-1 and MuRF-1 [115]. PI3K/Akt plays a dual role in protein turnover under hypertrophic stumuli, by activating mTOR to increase protein synthesis and by inhibiting FOXOs transcription factors to inhibit protein degradation.

Figure 6. Proposed scheme for Akt/FOXO-mediated regulation of protein accumulation in cardiac myocyte.



Hypertrophic stimuli phosphorylate Akt, and then Akt increases protein accumulation by increase protein synthesis (through mTOR) and decrease protein degradation through inhibit FOXO transcription factors, which activate atrogenes including atrogin-1, MurF-1. (Skurk C. 2005)

However, the exact roles of UPS in cardiac hypertrophy still remain to be determined, because there are inconsistencies between different studies. This is not surprising, because UPS may play different roles during different phases of development of hypertrophy and in different models of hypertrophy. It has been reported that the gene expression and protein abundance of proteasome subunits as well as proteasome activities

are increased during pressure overload-induced cardiac hypertrophy, and that proteasome inhibitors completely prevents cardiac hypertrophy[118]. Likewise, Razeghi et al. have reported that ubiquitin B, Atrogin-1, MurF-1 and the proteasome subunit PSMB4 transcript levels are increased during pressure overload-induced cardiac hypertrophy[119]. In contrast, Tsukamoto et al. have found that proteasome activities are depressed during pressure overload-induced cardiac hypertrophy and dysfunction, along with increased ubiquitination of protein [120]. Further studies are therefore needed to resolve the controversy concerning the role of UPS during cardiac hypertrophy.

Cyclic GMP-generating Agents and Cardiac Hypertrophy

In cardiomyocytes, cGMP is generated by soluble and particulate guanylyl cyclases. Natriuretic peptides activate particulate guanylyl cyclase[121]. Nitric oxide (NO), and to a lesser degree, carbon monoxide (CO), activate soluble guanylyl cyclase (sGC)[122,123].

The Natriuretic Peptide-cGMP Pathway and Cardiac Hypertrophy

Cardiac hypertrophy is characterized by re-expression of fetal genes, including the ANP and BNP genes, along with elevation of circulating levels of the ANP and BNP peptides[124]. It has been postulated that the functions of these circulating natriuretic peptides is to reduce the loading of the heart by increasing vasodilatation and decreasing body fluids [125].

Natriuretic peptides are not only circulating hormones, but also local autocrine and/or paracrine factors. Three natriuretic peptides have been identified: ANP, BNP, and C-type

natriuretic peptide (CNP). Normally, ANP is synthesized mostly in the atrium and BNP is synthesized mostly in the ventricle. ANP and BNP bind to natriuretic peptide receptor-A (NPR-A) and natriuretic peptide receptor-C (NPR-C), whereas CNP is the ligand of natriuretic peptide receptor-B (NPR-B) and NPR-C[126]. NPR-A is a plasma membrane guanylyl cyclase (GC) receptor, also known as guanylyl cyclase A (GC-A). ANP and BNP activate GC-A receptors expressed in a variety of tissues, thereby leading to the generation of cGMP in these cells as second messenger[121]. NPR-C is known as the "clearance receptor". Its primary action seems to clear the natriuretic peptides to modulate the local abundance of natriuretic peptides and their effects[127]

Expression of ANP and BNP (especially BNP) is upregulated in cardiac ventricles with pathological hypertrophy and heart failure, along with increases in the circulating levels of corresponding peptides[126,128,129]. Overexpressing ANP or GC-A in transgenic mice reduces cardiac hypertrophy[130,131]. ANP and BNP inhibit the hypertrophic effects of Ang II or norepinephrine (NE) via NPR-A and cGMP generation[132,133]. Conversely, using particulate guanylyl cyclase inhibitor, HS-142-1, blocking NPR-A has been shown to enhance the hypertrophic effects of PE and abolish the antihypertrophic effects of cGMP on cultured cardiomyocytes[132,134]. Inactivation of either *Nppa* (natriuretic peptide precursor A) or *Npr1* (encoding NPR-A) increases LVM; blood pressure is also increased in these knockout models, but the increased LVM is disproportionate compared to the increase in blood pressure[135,136]. To exclude cardiac hypertrophy induced by a secondary myocardial growth response due to increased afterload in germ-line GC-A or pro-ANP deletion, Holtwick et al. selectively inactivated the GC-A gene in cardiomyocytes. This selective loss of the GC-A receptor in the heart resulted in hypertrophic cardiac growth at

baseline along with hypotension. Moreover, afterload-induced hypertrophy was significantly greater in knockout animals than in their wild-type counterparts[137]. cGMP analogs and an inhibitor of cGMP specific phosphodiesterases suppress PE-induced increased protein synthesis[133,134]. In our laboratory, engineered transgenic mice expressing a constitutively activated guanylyl cyclase domain of the NPR-A in cardiomyocytes display increased intracellular cGMP in cardiomyocytes, and are protected from the hypertrophic effects of isoproterenol and abdominal aortic constriction[138]. Taken together, it appears that the ANP/BNP-GC-A-cGMP signaling pathway functions as cardiomyocyte intrinsic antihypertrophic factor in heart.

NO- cGMP Pathway and Hypertrophy

sGC are activated by NO, which is generated by nitric oxide synthases (NOS). There are 3 isoforms of NOS that are all expressed in heart [122]. NOS1 (neuronal NOS, or nNOS) and NOS3 (endothelial NOS, or eNOS) are constitutively expressed and regulated by Ca^{2+}, whereas NOS2 (inducible NOS, or iNOS) is not Ca^{2+}-regulated and is induced by stimulation of cytokines[139]. NO has been shown to attenuate cardiac hypertrophy, promote apoptosis and modulate cardiomyocyte contractility. NO stimulates sGC within cardiomyocytes and increases intracellular cGMP levels. Large amounts of NO attenuate myocardial contraction and cause tissue damage[140]. However, chronic administration L-arginine, the precursor of NO, to spontaneously hypertensive rats attenuates cardiac hypertrophy along with increased myocardial content of cGMP[141]. Yoshikazu Maeda et al. have shown that exogenous NO suppresses protein synthesis in cardiomyocytes, and adenovirus-mediated constitutive NOS3 gene expression in cardiomyocytes increases endogenous NO and inhibits increased cardiac protein synthesis induced by α-adrenergic

agonist, as well as increased cGMP contents of cardiomyocyte[142]. The studies of Kai et al. have indicated that NO donors and cGMP anologs suppress the hypertrophic effects of α-adrenergic agonist by activating protein kinase G I (PKG I), and endogenous PKG I mediates antihypertrophic effects of NO in a cell culture model of cardiomyocyte hypertrophy, but not its proapoptotic effects. Adenoviral overexpression of PKG I selectively enhances the antihypertrophic effects of NO donors and cGMP analogs, whereas it does not increase the susceptibility to apoptosis of NO[143]. NO has been shown to promote apoptosis in cardiomyocytes in a dose dependent manner, but this study has shown that NO induced apoptosis is by a caveolaecGMP-independent mechanism. Conversely, NOS3-/- mice display increased LVM and cardiomyocyte volume after pressure overloading even after normalizing blood pressure during the post-myocardial infarction period and pressure overload[144,145]. These studies show that NO can inhibit cardiac hypertrophy through cGMP-PKG I signaling pathway via activation of sGC.

Angiotensin-converting enzyme (ACE) inhibitors are used to treat cardiac hypertrophy in hypertensive patients. Some evidence shows that this antihypertrophic action is abolished by the B2-kinin receptor anagonist, indicating that increased bradykinin may play an important role in the antithypertrophic effects of ACE[146]. ACE is a bradykinin (BK) degrading enzyme, and ACE inhibitors increase the concentration of BK, which binds to B2 receptor in endothelial cells to increase NO level. NO stimulates sGC in cardiomyocytes, increasing cGMP content to exert antihypertrophic effects[147,148]. It has been shown that BK prevents Ang II-induced hypertrophy of isolated cardiomyocytes along with an increase in cytosolic concentration of cGMP; the antihypertrophic effects of

BK are abolished by NOS inhibitor and sGC inhibitor[148,149]. BK inhibits the hypertrophic effects of AngII in perfused whole rat heart associated with increased left ventricular cGMP concentration, and ACE inhibitor inhibits hypertrophy but this effect is abolished by B2-kinin receptor antagonist[148]. These studies support the notion that BK exerts antihypertrophic effects by increasing NO/cGMP signaling.

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), the cholesterol-lowering drugs, have been shown to inhibit cardiac hypertrophy through cholesterol-independent mechanisms by up-regulation of eNOS and inhibition of Rho A. [150,151]. It has been demonstrated that statins can improve endothelial function and stabilize eNOS mRNA in endothelial cells, leading to an increase in NO production by endothelial cells[152].

However, the effects of NO on cardiac phenotypes are complex, as it also has cGMP-independent effects. NO reacts with superoxide to generate peroxynitrite, a reactive oxygen species that is capable of triggering a series of cytotoxic processes and has been linked to cardiac remodeling and dysfunction[153]. Chronic heart failure is associated with enhanced expression of myocardial NOS2, which may exert different function in cardiomyocytes[154]. Mice with either NOS2-deletion and NOS2-overexpression mice have shown that NOS2 is detrimental and may increase mortality in heart failure[155]. NOS3-deficient mice display enhanced cardiomyocyte contractility, but NOS1-deficient mice exerted opposite effect. Both NOS1-/- and NOS3-/- mice develop age-related hypertrophy[156]. Myoglobin is able to effectively metabolize NO, and transgenic mice with concomitant myoglobin-deficiency and cardiac-specific overexpression NOS2 develop

cardiac hypertrophy[157]. NOS isoforms appear to play distinct roles in modulating cardiac structure. NOS3 localizes to caveolae, NOS1 is present in the sarcoplasmic reticulium (SR) and NOS2 is expressed in the cytosol. Differences in spatial confinement and levels of production may be responsible for complex and sometimes different effects of NO on cardiomyocytes[158].

Mechanisms of Antihypertrophic Effects of cGMP

The cGMP signaling pathway is essential for the hypertrophic effects of ANP/BNP, NO and BK, but what is the downstream of this pathway and how does it work?

In general, cGMP effectors include PKG, cGMP-gated cation channels, and cGMP-regulated phosphodiesterases. Two PKG genes have been identified, encoding for PKG I (including α- and β- splice variants) and PGK II. In cardiomyocytes, PKG I has been suggested to mediate the antihypertrophic effects of cGMP[159]. Inhibition of PKG I in cardiamyocytes enhances agonist-induced hypertrophy, while upregulation of PKG I inhibits hypertrophy[142,160].

Inhibition of Ca^{2+}-calcineurin-NFAT signaling

Numerous studies have recognized the importance of Ca^{2+}-sensitive signaling pathways in cardiac hypertrophy[161]. Calcium/calmodulin acts as an important second messenger for diverse signals including Ang II, ET-1, α-adrenergic agents and mechanical stretch which trigger cardiac hypertrophy [162]. Three calcium/calmodulin-dependent enzymes that have profound effects on cardiomyocytes are CaMK, the phosphatase calcineurin and myosin

light chain kinase (MLCK)[161]. It has been shown that cGMP/PKG inhibit LTCC by phosphorylating L-type Ca^{2+} channel (LTCC) or a closely associated protein[163]. Likewise, cGMP analogs reduce the myofilament response to Ca^{2+} in cardiomyocytes[164]. Due to the central role of Ca^{2+} in cardiac hypertrophy, cGMP may exert antihypertrophic effect by inhibiting both calcium and its downstream effects. In cardiomyocytes, activation of calcineurin by Ca^{2+} results in the dephosphoryation of the NFAT transcription factor, leading to its nuclear translocation and subsequently induction of hypertrophic gene expression[12]. It is well established that activation of the calcineurin/NFAT pathway is sufficient and necessary for the development of cardiac hypertrophy[13]. It has also been shown that calcineurin activation in cardiomyocytes depends on Ca^{2+} entry via LTCC[165] . Fiedler et al have shown that activation of PKG I suppresses single LTCC open and transient amplitude, thereby suppressesing NFAT transcriptional activity, BNP induction, and cell enlargement in response to α_1-adrenegic receptor agonist stimulation; however, it did not suppress the response to adenoviral expression of a Ca^{2+}-independtdent constitutively active calcineurin mutant, confirming that cGMP-PKG I signaling pathway inhibits calcineurin-NFAT signaling upstream of calcineurin[160]. CaMK is activated through an increase in Ca^{2+} during hypertrophic stimuli and CaMK inhibitors can suppress hypertrophy and fetal gene expression in adult cardiomyocytes[165]. Because cGMP decreases Ca^{2+} levels by inhibiting LTCC, it will also inhibit the downstream CAMK. MLCK phosphorylates MLC-2v, leading to higher sarcomere reorganization, which is a characteristic of cardiac hypertrophy. Phosphorylation of MLC-2v also increases sensitivity of the myofilaments to calcium [161].

cGMP, which inhibits LTCC, could thus downregulate MLCK, leading to inhibition of MLC-2v phosphorylation and its effects on sarcomere reorganization.

Downregulation of MLP

The cytoskeleton-associated muscle LIM protein (MLP) is a member of the LIM-only class of the LIM domain protein family which is highly expressed at the level of Z disk in striated muscle cells[166]. ET-1-induced cardiomyocyte hypertrophy is associated with an increase in the level of MLP expression, and antisense downregulation of MLP suppresses cardiomyocyte hypertrophy. Likewise, overexpression of MLP by itself increases cardiomyocyte size and sarcomere organization. NO can downregulate expression of MLP in ET-1 stimulated cardiomyocytes, as well as inhibit protein synthesis. MLP may act as scaffold protein to facilitate sarcomere assembly and cell enlargement[155]. Therefore, MLP downregulation may be one important mechanism contributing to the antihypertrophic effects of cGMP/GC-A signaling pathway.

Inhibition of Rho A/Rho kinase signaling

The Rho proteins belong to the family of small G proteins, and recent studies have demonstrated that RhoA is involved in the pathogenesis of cardiac hypertrophy[38,39,43]. Rho A and its effector Rho-kinase are key regulators of the organization of myofibrils and sarcomeric protein[167]. PKG I phosphorylates and inhibits RhoA as well as the translocation of RhoA to the membrane, inducing disassembly of the actin cytoskeleton[168]. Further studies have demonstrated that cGMP inhibits the transcriptional activation of SRF by inhibiting upstream RhoA activators, RhoA and downstream RhoA effectors in smooth muscle cells[169]. These studies suggest that cGMP/PKG signaling pathway exert antihypertrophic effects in part via inhibition of RhoA signaling.

Inhibition of NHE

The NHE represents one of key components to maintain physiological intracellular pH in heart cells. NHE activity is increased during the development of cardiac hypertrophy, and cariporide (a NHE inhibitor) prevents cardiac hypertrophy[2,44,45]. NO donors, cGMP analogs, and ANP all inhibit NHE activity in cardiomyocytes[170,171].

Inhibitton of the MAPK signaling pathway

Sharma et al. have shown that overexpression of the ANP receptor-A inhibits the activity and abundance of ERK2 and P38MAPK in human vascular smooth muscle cell stimulated by Ang II and platelet-derived growth factor (PDGF); it also stimulates MAPK-phosphatase-3, and thus presumably counter-regulates MAPKs [172]. It has also been shown that cGMP inhibits epidermal growth factor (EGF)-induced activation of MAPK in baby hamster kidney (BHK) cells expressing activated PKG, but not in PKG-deficient and inactive PKG cells. The inhibition of cell growth by cGMP depends on PKG activation, which phosphorylates c-Raf, leading to the uncoupling of the Ras-Raf kinase interaction and the induction of the MAPK phosphatase expression to inhibit MAPK activity[173]. These studies suggest that cGMP/PKG signaling may exert antihypertrophic effects by inhibiting MAPK activity.

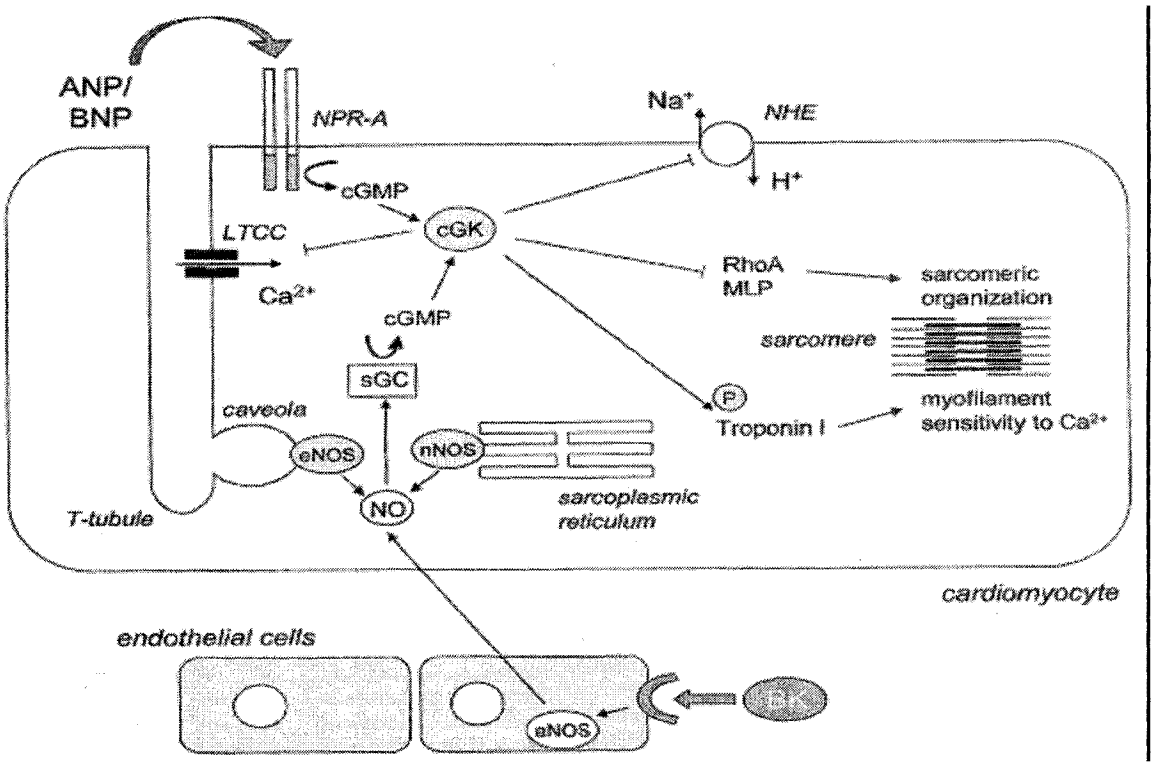
Inhibition of TnI

PKG I interacts with cardiac Troponin T (TnT) and phosphorylated TnI; however, since PKG I does not interact with TnI, it is likely that TnT functions as an anchoring protein for PKG I to phosphorylate TnI, leading to reduced responsiveness of myofilaments to intracellular Ca^{2+}[174,175]. It has also been suggested that PKG I phosphorylates the

vasodilator-stimulate phosphoprotein (VASP), which control cytoskeletal actin filament assembly to mediate the effect of cGMP on cardiomyocyte size[143].

Overall, evidence indicates that cGMP may exert antihypertrophic effects through multiple pathways. The figure 7 summarizes the generation and possible action sites of cGMP.

Figure 7. Generation of cGMP within cardiomyocytes and downstream effects



The natriuretic peptides ANP and BNP can generate cGMP within cardiomyocytes upon binding to the NPR-A receptor. Cyclic GMP can also be produced by generation of NO by either eNOS (located within caveolae of cardiomyocytes) or nNOS (located in the vicinity of the sarcoplasmic reticulum). NO is also produced by eNOS in neighboring endotheolial cells upon stimulation with BK. Cyclic GMP within cardiomyocytes will activate cGKs(PKGs). This will in turn inhibit the activity of LTCC or that of NHE. Activated PKG will also inhibit Rho A and MLP; both molecules are involved in the reorganization of sarcomeric proteins, which is a prominent feature of LVH. Activated PKG will also phosphorylate troponin I, which results in decreased sensitivity of myofilaments to intracellular Ca^{2+}. Abbreviations are as described in the text. (Deschepper C. F. Vascular Disease Prevention 2005)

Given the evidence showing that increased cGMP is associated with protection against hypertrophy in vitro and in vivo, it may be possible in the future to exploit this pathway to design new therapeutic approaches. ACE inhibitors have been shown to have effective antihypertrophic effect, presumably via the BK-NO-cGMP signaling pathway. Statins, in addition to their cholesterol-lowering effects, also have antihypertrophic effects. In clinical trials in patients with acute decompensated heart failure, nesiritide, a recombinant form of human BNP, has shown short-term hemodynamic and symptomatic improvements [176]. The concept that increasing NOS activity, reducing ANP degradation, and increasing GC-A activity by increasing the concentration of agents that stimulate cGMP/PKG or by activating molecules that are targets of this pathway may all provide new therapeutic avenues to treat hypertrophy.

The effects of cGMP on protein synthesis in cultured cardiomyocytes

Isolated cardiomyocytes in culture are frequently used to study the effects of cGMP on protein synthesis under different hypertrophic stimuli. Both neonatal cardiomyocytes and adult cardiomyocytes have been used, but they are different in several respects. Concerning cell morphology and size, neonatal cardiomyocytes are round-up or spindle-shaped, while adult cardiomyocytes are rod-shaped with a 10 times bigger volume[177]; concerning metabolism, the basal protein synthesis rate of adult cardiomyocytes is almost idential to the rate observed in vivo, but the rate of neonatal cardiomyocytes is 6-fold higher[178]. Neonatal and adult cardiomyocytes also differ in term of the density and nature of receptor, as well as in the expression of protein level.[179,180,181]. In particular, the

expression of PKG I mRNA and protein levels are much higher in neonatal cardiomhocytes than in adult cardiomyocytes[159]. In term of responses to hypertrophic stimuli, neonatal cardiomyocytes develop hypertrophy and hyperphasia, while adult cardiomyocytes are terminal differentiated cells and do not undergo mitosis[182]. Cardiac hypertrophy develops under pathophysiological conditions in adult, so the cultured adult cardiomyocytes are most likely to reflect the in vivo situation. However, the adult cardiomyocyte culture is more difficult to establish and is used less frequently.

The inhibitory effect of cGMP on protein synthesis has been studied normaly in cultured neonatal cardiomyocytes. Calderone et al. have shown that ANP and NO donor, S-nitroso-N-acetyl-D, L-penicillamine (SNAP) inhibit norepinephrine (NE)-stimulated protein synthesis evaluated by [^{3}H]Leccine incorporation in cultured neonatal rat cardiomyocytes, accompanied by increased intracellular cGMP levels. 8-bromo-cGMP mimics this growth-suppressing effect. They further suggest that cGMP-mediated inhibition of NE-stimulated Ca^{2+} influx plays a major role in this process[133]. A low density (100-200 cells/mm^2) cell culture is used in the above experiment, but the inhibitory effect of cGMP is absent when neonatal cardiomyocytes are cultured at a higher density (1000 cells/mm^2), as shown by Handing et al[183]. This suggests that cell density exerts qualitative effects on the pathways which mediate α-adrenergic agonists-stimulated protein synthesis in neonatal cardiomyocytes[184]. Horio et al. have shown that HS-142-1 (a particulate guanylyl cyclase inhibitor) increases basal and PE-stimulated protein synthesis in a concentration-dependent manner in cultured neonatal cardiomyocytes; ANP, zaprinast (a cGMP-specific PDE inhibitor) and 8-bromo-cGMP

suppressed PE-stimulated protein synthesis evaluated by [^{14}C] phenylalaine incorporation[134]. Maeda et al. have demonstrated that NO donors, and 8-bromo-cGMP cause concentration-dependent decreases in PE-stimulated protein synthesis in neonatal cardiomyocytes; endothelial constitutive NO synthase (ecNOS) gene transfer significantly increases cGMP content in cardiomyocytes and inhibits PE-stimulated protein synthesis; moreover, this inhibition is abolished by the presence of a NOS inhibitor[142]. It has also been shown that 8-pCPT-cGMP modestly attenuates PE-stimulated protein synthesis in neonatal cardiomyocytes and completely suppressed PE-stimulated protein synthesis in neonatal cardiomyocytes overexpressing PKGIβ without promoting apoptosis. This study also implies that effects of NO on apoptosis is PKG-independent[143].

There are also some studies that have demonstrated the inhibitory effect of cGMP on protein synthesis in cultured adult cardiomyocytes. Rosenkranz et al. have shown that all three natriuretic peptides (ANP, BNP and CNP) prevent AngII-stimulated protein synthesis in cultured adult cardiomyocytes, and HS-142-1 blocks the inhibitory effects of all three natriuretic peptides. 8-bromo-cGMP mimics this inhibitory effect on adult cardiomyocytes. A cGMP-dependent protein kinase inhibitor, KT5823, abolishes the inhibitory effects of BNP and CNP, but not ANP, and this suggests that ANP prevents AngII-stimulated protein synthesis via both cGMP-dependent and independent mechanisms.[132] The same group has also shown that bradykinin inhibits AngII-stimulated protein synthesis and this effect is abolished by a guanylyl cyclase inhibitor in adult cardiomyocytes cultured with bovine aortic endothelial cells. This study supports the notion that bradykinin stimulates endothelial cells to release NO, inducing cGMP production in cardiomyocyte which prevents hypertrophy.

What is the specific in my project?

Cultured adult rat cardiomyocytes:

We chose adult cardiomycytes because these cells are more likely to reflect the cardiac hypertrophy developed in vivo[177,178,182-186]. Rats were used because rat hearts yield cardiomyocytes that are 10 times more numerous and more robust in culture than mouse hearts. In this in vitro model, selected growth-stimulus can be studied specifically and influence from other cell types and other neuroendocrine and mechanical factors can be excluded.

PE used as hormonal stimulus:

Catecholamines mediate cardiac hypertrophic effect mostly (if not all) by α_1-adrenergic receptors, for which PE constitutes a preferential agonist[187]. Further, it has been shown that PE, as well as NE, ET-1, Ang II, prostaglandin F2a and pressure-overload induce cardiac hypertrophy via $G_{\alpha q}$ -mediated mechanism and PE is considered as a prototypical activator of $G_{\alpha q}$-coupled receptors[24,25].

SECTION 2: PROJECT OUTLINE AND HYPOTHESIS

Increased protein accumulation plays an important role in the development of cardiac hypertrophy, which is recognized as a maladaptive response and a powerful independent risk factor for cardiovascular morbidity and mortality. cGMP has recently emerged as an endogenous molecule that inhibits cardiac hypertrophy and its maladaptive components, but its role in regulating protein accumulation during cardiac hypertrophy is still remaining to be explored. We hypothesized that cGMP prevents increased protein accumulation by inhibiting positive regulations of protein synthesis or by stimulating positive regulation of protein degradation. These include S6K1, ribosomal S6 protein, ERK, 4E-BP-1, UBF, Atrogin-1 and MurF-1, which all have been shown to be regulated by PE in cardiomyocytes *in vitro*.

We first studied the effects of 8-bromo-cGMP on PE-induced increased accumulation of newly synthesized proteins in adult rat ventricular cardiomyocytes (ARVC) by [^{3}H] phenylalanine incorporation. Then we studied the effects of 8-bromo-cGMP on S6K1, ribosomal S6 protein, ERK, 4E-BP-1 phosphorylation by western blot analysis. Furthermore, we investigated the effects of 8-bromo-cGMP on protein degradation. We used Q-PCR to determine mRNA expression of Atrogin-1 and MurF-1, which are the E3s involved in protein degradation that have recently received more attention.WE also tested the effect of 8-bromo-cGMP on ROS generation during PE stimulation by chemiluminescence.

SECTION 3: MATERIALS AND METHODS

Animals

All procedures on animals were approved by the Institut de Recherches cliniques de Montreal (IRCM) Institutional Animal Care Committee and conducted according to guidelines issued by the Canadian Council on Animal Care. The nomenclature of the strain is in compliance with the recommendations of the International Rat Genetic Nomenclature Committee. WKY/Cfd rats originated from a colony maintained at the IRCM and derived from WKY/Cr parent from Charles River (St Constant, Canada). All the animals were used at 10-14 weeks old for these studies.

Materials

Cell culture medium M199 modified, L-ascorbic acid, Creatine, Carnitine, Taurine, Phenol-red, $NaHCO_3$, Glucose, 8-bromo-cGMP, PE, Hoechest 33258, Pepstain, Antipain, Leupeptin, PMSF, Na_3VO_4, NaF, Glycerol, NP-40, Thyroglobuline, Phenol solution, N-Lauroylsarcosine sodium, α-Propanol, NADPH, Lucigenin, Tiron and Cyclosporin A were purchased from Sigma Aldrich (Toronto, Canada); Tautomycin was from Calbiochem (San Diego, USA). Guanidine isothiocyanate, Tris, Dithiothreitol, FBS, EGTA, EDTA, HEPES buffer solution, Penicillin-Streptomycin, NaCl, Oligo (dT), RNase inhibitor, Reverse transcriptase, DEPC water and $TE_{0.1}$ were obtained from Invitrogen Life Technologies (Saint-Hyacinthe, Canada); Scintisafe Econo I, Trichloroacetic acid (TCA), SDS, KCl, KH_2PO_4, $MgSO_4$, Sodium citrate, Sodium acetate, Chloroform, Iso-Amyl alcohol and Lithium chloride were supplied by Fisher Scientific (Ottawa, Canada); Fatty acid-free Bovine Serum Albumin, Dnase I, Typsin and

Hyaluronidase were provided by Roche Diagnostics (Laval, Canada); BSA was from Serologicals Proteins Inc (Norcross, USA). Collagenase Type 2 was purchased from Worthington Biochemical Corporation (Freehold, USA); [^{3}H] Phenylalanine was from GE Health Care (Piscataway USA); Tween-20 was from Acros Organics (New Jersey, USA); Triton-X 100 was from ACP Chemical Inc. (Montreal, Canada). Laminin was from VWR international (Mississauga, Canada); Anti-S6K antibody, Anti-phospho-p70S6 kinase (Thr421/Ser424) antibody, Anti-S6 ribosomal protein antibody (Rabbit mAb), Anti-phospho-S6 ribosomal protein (Ser240/244) antibody, Anti- P42/44 MAPK antibody, Anti-phospho-P42/44 MAPK (Thr202/Tyr204) antibody, Anti-4E-BP-1 antibody, Anti-phospho-4E-BP-1 (Thr37/46) antiobody and Prestained protein marker were purchased from Cell Signaling (Beverly, USA); Anti-UBF antibody and Anti-phospho-UBF (Ser388) antibody were from Santa Cruz Biotechnology (Santa Cruz, USA); Goat anti-rabbit IgG was purchased from Chemicon International (San Francisco, USA); PMSF membrane and chemiluminescence HRP substrate were from Millipore Corporation (Bedford, USA); SYBR kit and Taq DNA polymerase were from QIAGEN (Toronto, Canada). Bio-Rad protein assay and Mini Trans-Blot Cell were from Bio-Rad Laboratories Inc. (Toronto, Canada); TD-700 fluorometer was from Sequoia-Turner (Sparks, USA); UV-visible recording spectrophotometer was from Shimadzu (Columbia, USA).

Isolation and culture of adult rat ventricular cardiomyocytes (ARVC)

Ventricular cardiomyocytes were isolated from hearts of adult rats as described previously[188]. Briefly, the rats were anesthetized with 1 ml of Xylazine and 2 ml of Ketamine hydrochloride. After falling asleep, the rats were disinfected on the thorax and

abdomen with 70% ethanol. The hearts were taken out and transferred to a Petri containing 15 ml of Krebs-Henseleit (KH) (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.19 mM, $NaHCO_3$ 25 mM, Glucose 11mM, KH_2PO_4 1.0 mM) + $CaCl_2$ (10mM) + heparin. After installing the hearts on the perfusion needle of the Langendorff perfusion system, the aortas were clamped by attaching the needle with a thread. The hearts were perfused with KH+ $CaCl_2$ for min, with KH for another 5 min, and then perfusion digestion solution (Collagenase type 2 80 mg, Hyaluronidase 24 mg, BSA 80 mg in 80 ml KH solution) for 20 min (the solution being recycled during the last 15 min). After removing the atria and the aorta, the perfused ventricles were transferred to a Petri containing 15 ml incubation digestion solution (perfusion digestion solution supplemented with trypsin 0.2 mg/ml and DNase I 0.2 mg/ml). After 10 min of incubation at 37 °C, the cell suspensions were filtered with a 200 μm filter, and then were centrifuged at 1000 rpm for 1min. The pellets were resuspended with 10ml washing buffer (KH 22.5 ml, M199 22.5 ml, FBS 5 ml), the procedure was repeated twice, and then the cells were layered on 6% BSA (0.6 g BSA in 10 ml M199) to separate the cardiomyocytes from non-cardiomyocytes. The supernatant was discarded and and the cells were resuspended in M199 media ($NaHCO_3$ 26.2 mM, HEPES buffer solution 0.02 M, BSA 0.2%, Pen-Strep1%, L-ascorbic acid 0.1 mM, Creatine 5.0 mM, Carnitine 2.0 mM, Taurine 5.0 mM, phenol-red) + 10% FBS. The cells were counted with a hemacytometer, then seeded in 6-well laminin-coated dishes (with M199+10%FBS+2% Pen-Strep) at an approximate density of $3\text{-}4x10^4$/well. After allowing the cells to attach in the incubator (95% air/5% CO_2) for at least 2 hours, the media was then changed to the supplemented serum-free medium. After 16-18 h of incubation, the cells were used for experiments.

[^{3}H] phenylalanine incorporation

Accumulation of newly synthesized proteins within cardiomycytes was assessed by incorporation of [^{3}H] phenylalanine into proteins, as described previously, with modifications[188]. Briefly, the cells were treated with PE (10^{-5} M) for indicated times (for time course experiments), or were treated with PE (10^{-5} M), cGMP (10^{-4} M) or PE (10^{-5}M) + cGMP (10^{-4} M) (cGMP was added 30 min before the stimulation with PE in the PE + cGMP groups) in addition of [^{3}H] phenylalanine (1 μCi/ml). In control wells, only [^{3}H] phenylalanine (1 μCi/ml) was added. After incubating the cells in 95% air/5% CO_2, the cardiomyoctytes were washed with ice-cold PBS to terminate the [^{3}H] phenylalanine incorporation. 10% TCA was added in each well for 30 min at 4°C to precipitate the proteins. The precipitated proteins were dissolved in 1% SDS at 37°C for 60 min (500 μl per well). Radioactivity was measured by liquid scintillation counting (LKB Wallac 1215 RacBeta liquid scintillation counter) by using 400 μl of sample, and the remainder was used for DNA quantification. To quantify DNA, 50 μl from each of sample was added to 2 ml of a solution of Hoechst 33258 (0.1 μl/ml) in TNE. Fluorescence (350nm excitation, 450nm emission) was measured in a TD-700 fluorometer.

Sample preparation for western blot analysis

After treating cells for the indicated time, the cells were washed with ice-cold PBS and homogenized in lysis buffer (Tris-HCl PH=7.5 50 mM, EDTA 1 mM, EGTA 1 mM, Na_3VO_4 1 mM, NaF 50mM, sucrose 270 mM, 1% triton-X 100, leupeptin 5 μg/ml, pepstatin 5 μg/ml, antipain 5 μg/ml, DTT 1 mM, PMSF 200 μM). The total cell homogenates were centrifuged at 14,000 rpm in 10 min to remove nuclei. The supernatant

was aliquoted and the total protein concentration was determined by the Bradford method[188]. An aliquot containing 30-50 μg protein was used for western blot analysis.

NADPH oxidase assay

NADPH oxidase activity were carried out by lucigenin-enhanced chemiluminescence[189]. The adult rat ventricular cardiomyocytes (ARVC) were treated with PE (10^{-5} M) for indicated times (for time course experiments), or were treated with PE (10^{-5} M), cGMP (10^{-4} M) and PE (10^{-5} M) + cGMP (10^{-4} M) (cGMP was added 30 min before the stimulation in PE and PE + cGMP groups) or media alone for 15 min. The cells were washed with ice-cold PBS, and then scraped in 1 ml of PBS. The cells were then centrifuged at 2,500 rpm for 10 min at 4 °C, suspended in lysis buffer (KH_2PO_4 PH 7.4 20 mM, EGTA 1 Mm, leupeptin 5 μg/ml, antipain 5 μg/ml, pepstatin 5 μg/ml) and homogenized with 50 strokes in a glass homogenizer on ice. Proteins concentration was determined using the Brandford assay as described above and aliquots of 50 μg of protein were used for assay. NADPH oxidase activity was measured in phosphate buffer (KH_2PO_4 PH 7.4 50 mM, EGTA 1 mM and sucrose 150 mM), lucigenin (500 μM) as the electron acceptor, 100 μM NADPH as the substrate (final volume, 225 μl) and with or without inhibitor Tiron (10mM). Luminescence measured with luminometer was integrated over 3 s intervals for a total of 3 min at room temperature. A buffer blank was subtracted from each reading before transformation of the data.

Nuclear extraction

ARVC were washed with PBS, scraped in 400 μl swollen buffer (Tris PH 7.9 10 mM, KCl 10 mM, $MgCl_2$ 1 mM and DTT 1 mM) and incubated on ice for 1 h. Nuclei were

pelleted by centrifugation at 900 g for 10 min at 4 °C. The pellets were suspended in 400 µl homogenized buffer (Tris PH 7.9 10 mM, sucrose 300 mM, $MgCl_2$ 1.5 mM, DTT 1 mM, Triton X-100 0.3 %) for 10 min on ice, homogenized with 50 strokes in a glass homogenizer and centrifuged again as above. The pellets were resuspended in 40 µl 1x storage buffer (HEPES PH 7.5 10 mM, NaCl 300 mM, KCl 50 mM, DTT 1 mM, PMSF 1 mM and glycerol 20 %) and vortexed for 30 min at 4°C. The extracts were centrifuged at 13,000 g for 10 min at 4°C. The resulting supernatant was the nuclear extract. Proteins concentration was determined by Bradford assay and aliquots of 50 µg of protein were used for detecting UBF by Western blot.

Western Blot analysis

Samples containing 50 µg ofproteins were separated on SDS-polyacrylamide gels (10% for S6K1, 12% for ribosomal S6 protein and 4E-BP-1, 8% for UBF) and electrophoretically transferred to PMSF membrane using a Mini Trans-Blot Cell. Membranes were incubated in a blocking buffer (TBS 1X, 5% nonfat dry milk and 0.1% Tween 20) for 1 hour. Membranes were probed with first antibody (1:4000 dilution in TBS 1X, 0.1% Tween 20, 5% FBS) overnight at 4°C, then washed 4 times (5 min for each time) with washing buffer (TBS 1X, 0.1% Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution in TBS 1X, nonfat dry milk 5% and 0.1% Tween 20) for 1 h at room temperature. After washing 4 times (5 min for each time) in washing buffer, the membranes were detected by ECL. ImageQuant 5.0 software (Molecular Dynamics) was used for quantification of bands.

RNA extraction

The cells were washed with sterilized PBS at room temperature, then denatured in 3 ml denaturing solution (guanidium thiocyanate 4 M, sodium citrate 25 mM and 0.5% N-lauroylsarcosine) and homogenized with a syringe. To the 3 ml homogenate, 0.3 ml sodium acetate (2 M), 3 ml water-saturated phenol and 0.6 ml chloroform/isoamyl were added and incubated on ice for 15 min, then centrifuged at 9,000 rpm for 20 min at 4°C. The upper aqueous phase was recovered without touching the interface (containing DNA and proteins), transferred to a fresh tube and added to an equal volume of isopropanol. Following incubation for 20 min at -20°C, the samples were centrifuged at 9,000 rpm for 60 min at 4°C. After discarding the supernatant, the RNA pellet was resuspended in 800 µl Lithium chloride (4M) and centrifuged at 9,000 rpm for 10 min at 4°C. Discarding the supernatant, the pellet was resuspended in 400 µl $TES_{0.1}$ and added with 400 µl chloroform. After centrifuging for 2 min, the aqueous phase was recovered and precipitated with NaOAC 3M (pH 5.2) and 100% ethanol overnight at -80°C. After centrifuging at 14,000 rpm for 10 min at 4°C, the RNA pellet was washed with 75% ethanol, and dried with a speed vac. The RNA pellet was dissolved in DEPC water and was quantified with spectrophotometer.

Reverse transcription

1.5 µg RNA from each sample was mixed with 1 µl Oligo dT (0.5 µg/µl) and DEPC water to reach a final volume to 11 µl, then heated at 70°C for 10 min and chilled on ice. The RxMix (5x First strand buffer 4 µl, 1M DTT 2 µl, dNTP 10mM 1 µl and RNA guard 1 µl) was added to the previous 11 µl, mixed and incubated at 42 °C for 2 min, then 1 µl

of reverse transcriptase was added and incubated for another 50 min at 42 °C. After heating at 70°C for 15 min for extension, the cDNA was kept at 4 °C (stored at -20 °C).

PCR

cDNA products were amplified by PCR with different primers to verify cDNA quality and primer design. cDNA was diluted to 1/20 in DEPC water. 1 µl of diluted cDNA was used for PCR; 0.5 µl of RNA was used for PCR to check for eventual DNA contamination during RNA extraction. 1 µl of cDNA or 0.5 µl of RNA were mixed with Master Mix (dNTP 10mM 0.2 µl, primer 2 µl, 5x Q-solution 2 µl, 10xPCR buffer 1 µl, DEPC water 3.7 µl and Taq polymerase 0.1 µl). Primers used were as following: rat UBF forward: 5'-CCCAAACCCCAAGTCTAGC-3', reverse: 5'-TCCTCTTCCTCCTCGTCATC-3'; rat MR-1 forward: 5'-AGAACCCCATGAAAGCTGTG-3', reverse: 5'-ACACGGTCCTTGTCCACTTC-3'; rat Atrogin-1 forward: 5'-GACCGGCTACTGTGGAAGAG-3', reverse: 5'-AATCCAGCTGCCCTTTGTC-3'; rat MuRF-1 forward: 5'-GAGCTGCCCACTTCCTTTC-3', reverse: 5'-TTTGGGGATGGGTACTGAAG-3'; rat Hprt forward: 5'-TGTTGTTGGATATGCCCTTG-3', reverse: 5'-CCGCTGTCTTTTAGGCTTTG-3'. Primers were designed to be compatible with a single PCR thermal profile (95 °C for 10 min, and 34 cycles of 94 °C for 1 min, 58 °C for 45 s, and 72 °C for 45 s) so that multiple transcripts could be analyzed simultaneously. PCR was performed on MJ PTC-225 Thermal Cycler (GMI Inc., Ramsey, USA).

The PCR products were checked by agarose gel electrophoresis and visualized by AlphaImager™ 2200 (Alpha Innotech Inc., San Leandro, USA).

Q-PCR

Q-PCR was performed in duplicate on a MX 3005 (STRATAGNENE, La Jolla, USA) using SYBR Green as a double-stranded DNA-specific dye according to manufacture's instruction. 2 μl diluted cDNA (1:200 for MuRF-1 and Atrogin-1; 1:100 for UBF and MR-1) was mixed with Q-PCR Mix (primer 2μl, SYBR Green 10 μl and DEPC water 6 μl). Q-PCR thermal profile: 95 °C for 15 min for 1 cycle, 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for 40 cycles. Post-amplification dissociation curves were assessed to verify the presence of a single amplification product. The selected genes were normalized to Hprt to standardize the quantification. MxPro software was used for analyzing Q-PCR. Fold changes in gene expression were determined using the C_t method.

Statistical analysis

Data are presented as mean ± SD for the number of independent experiments indicated. The *t* test was used to determine significant differences between two groups. One-way analysis of variance (ANOVA) was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the *t* test. $P< 0.05$ was considered to be significant.

SECTION 4: RESULTS

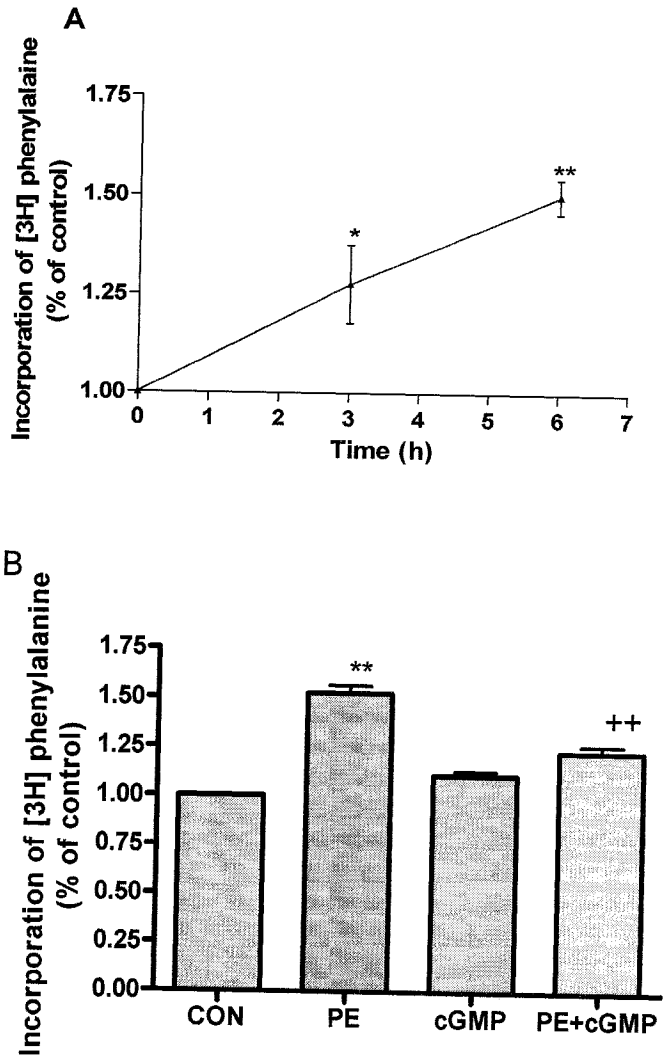
The effects of cGMP analog on PE-induced accumulation of newly synthesized proteins in ARVC

The experiments in this project were conducted on isolated ARVC because the growth properties of these cells are considered to be more relevant to cardiac hypertrophy developed in adult hearts[190]. We have used rats instead of mice as a source of cardiomyocytes because rat hearts yield 10 times more cells than mice hearts, as well as being more robust in culture[191].

To optimize the time of incubation, we first stimulated ARVC with PE (10^{-5} M) for 3 h and 6 h. The addition of PE to cultured ARVC for 3 and 6 h caused an increase in [^{3}H] phenylalanine incorporation by 28 % ($p<0.05$; n=6) and 50% ($p<0.001$; n=6), respectively (Fig. 8A). According to the PE time course, we chose 6 h PE-stimulation for further investigation.

Then we investigated the effect of 8-bromo-cGMP on PE-stimulated protein synthesis in ARVC. The addition of 8-bromo-cGMP alone (10^{-4} M) had no effect on basal [^{3}H] phenylalanine incorporation, but attenuated the PE-mediated increase in [^{3}H] phenylalanine incorporation by 57.1% ($p<0.05$; n=12) (Fig. 8B). This result demonstrates that cGMP analog suppressed the effect of PE on accumulation of newly synthesized proteins, which is a characteristic of cardiac hypertrophy under α-adrenergic agonist PE stimulation in ARVC.

Figure 8. 8-bromo-cGMP suppresses PE-increased protein synthesis in cultured ARVC.



(A) Cultured ARVC were incubated with PE (10^{-5} M) for 3 h and 6 h. Protein accumulation was assessed by measurement of [^{3}H] phenylalanine incorporation into TCA- precipitatable material. Protein accumulation rate increased 28% within 3 h ($p<0.05$; n=12) and 50% ($p<0.001$; n=12) within 6 h. (B) 8-bromo-cGMP inhibited PE-stimulated [^{3}H] phenylalanine incorporation. Cultured ARVC were incubated with PE, 8-bromo-cGMP, PE + 8-bromo-cGMP or media alone for 6 h. 8-bromo-cGMP suppressed PE-increased [^{3}H] phenylalanine incorporation by 57.1% ($p<0.05$; n=12). Data are mean ± SD. * $p<0.05$ *vs.* basal, ** $p<0.001$ PE *vs.* Basal or con, ++ $p<0.001$ PE + cGMP *vs.* PE.

PE stimulates ROS production in ARVC

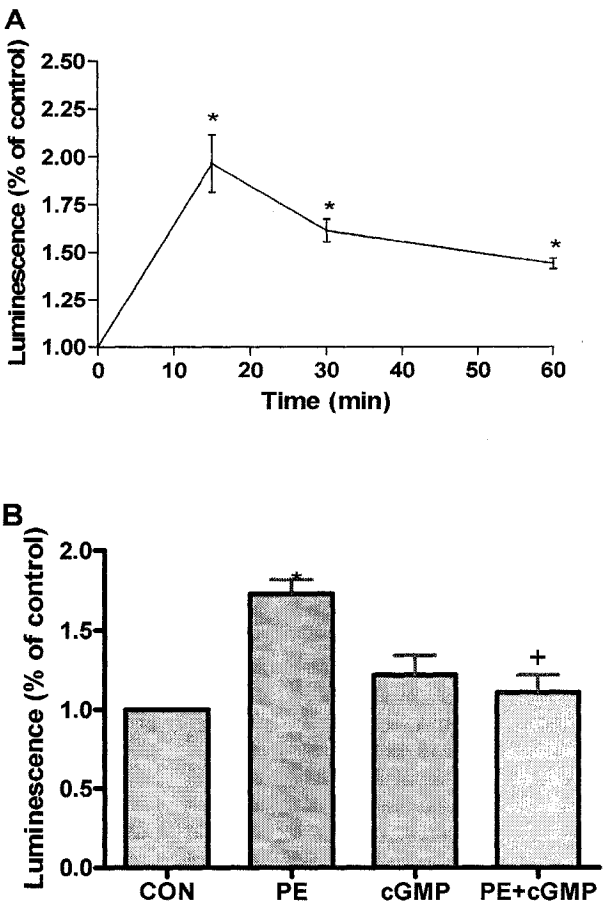
ROS play an important role in GPCR induced cardiac hypertrophy by activating multiple signaling pathways and transcription factors[55-62]. The NADPH oxidase system is one of the most important oxidase system in smooth muscle[189]. ROS production was assessed by NADPH oxidase activity in cultured ARVC with lucigenin-enhanced chemiluminescence assay. We prepared cell homogenates and assayed NADPH oxidase activity to examine the activity of specific intracellular oxidase to eliminate any potential barrier to lucigenin entry presented by the plasma membrane.

In ARVC exposed to PE (10^{-5} M) for 15 min, 30 min and 60 min, NADPH-dependent O_2^- production was increased by 99% ($p<0.01$; n=5), 56% ($p<0.01$; n=5) and 44% ($p<0.01$; n=5), respectively, as assessed by lucigenin-enhanced chemiluminescence (Fig. 9A). The subsequent addition of tiron (10 mM) caused a decrease in chemiluminescence to the baseline value. The peak response was at 15 min and we then used 15 min stimulation for further investigation.

cGMP analog blocks PE-stimulated ROS production

We further investigated the effect of 8-bromo-cGMP on PE-stimulated ROS production in cultured ARVC. The ARVC were incubated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 15 min. The addition of 8-bromo-cGMP had no effect on basal ROS production assessed with lucigenin-enhanced chemiluminescence assay, but blocked the PE-mediated increase in ROS production ($p<0.01$ PE +8-bromo-cGMP *vs.* PE; n=5 and $p>0.05$ PE + 8-bromo-cGMP *vs.* con) (Fig. 9B). This result demonstrates that cGMP analog blocks PE-stimulated ROS production.

Figure 9. 8-bromo-cGMP blocks PE-stimulated ROS production in ARVC.

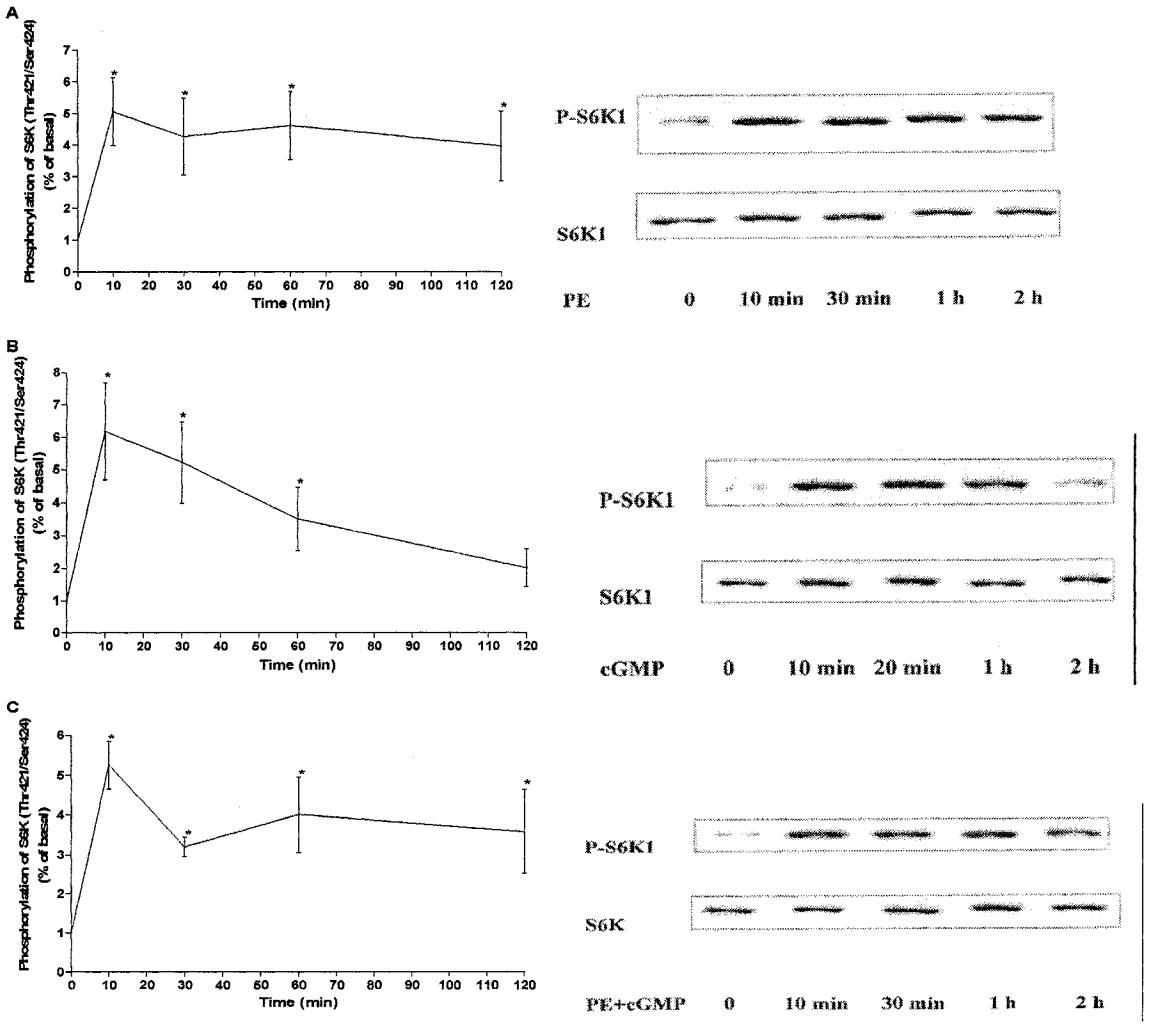


(A) ARVC were exposed to PE (10^{-5} M) for 15 min, 30 min and 60 min, washed and homogenized. NADPH oxidase activity was measured in phosphate buffer, lucigenin (500 μM) as the electron acceptor, 100 μM NADPH as the substrate (final volume, 225 μl) and with or without inhibitor Tiron (10mM). Luminescence measured with luminometer was intergrated over 3 s intervals for a total of 3 min at room temperature. In ARVC exposed to PE for 15 min, 30 min and 60 min, NADPH-dependent O_2^- production was increased by 99% ($p<0.01$; n=5), 56% ($p<0.01$; n=5) and 44% ($p<0.01$; n=5), respectively, as assessd by lucigenin-enhanced chemiluminescence. The subsequent addition of tiron (10 mM) caused a decrease in chemiluminescence to the baseline value. The peak response was at 15 min. (B) The ARVC were incubated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 15 min. The addition of 8-bromo-cGMP had no effect on basal ROS production assessed with lucigenin-enhanced chemiluminescence assay, but blocked the PE-mediated increase in ROS production ($p<0.01$; PE + 8-bromo-cGMP *vs.* PE; n=5 and $p>0.05$ PE + 8-bromo-cGMP *vs.* con). Data are the means ± SD of five independent experiments. * $p<0.01$ *vs.* basal or con, + $p<0.01$ *vs.* PE.

cGMP analog does not inhibit PE-induced Thr421/Ser424 phosphorylation on S6K1 in short term treatment

S6K1 is an important regulator of protein synthesis and phosphorylated S6K1 increases efficiency and capacity of protein synthesis during cardiac hypertrophic stimuli [94,95]. To study whether PE phosphorylates Thr421/Ser424 on S6K1, we treated ARVC with PE (10^{-5} M) for different times. Significant phosphorylation of S6K1 (Thr421/Ser424) was observed during the period ranging from 10 min to 2 h after stimulation, and reached a maximum at 10 min as assessed by Western blot analysis ($p<0.05$, $n=3$) (Fig. 10A). 8-bromo-cGMP (10^{-4} M) alone also significantly increased S6K1 (Thr421/Ser424) phosphorylation ($p<0.05$, $n=3$), although phosphorylation appeared to return to baseline at 120 min after stimulation (Fig. 10B). The addition of 8-bromo-cGMP did not inhibit PE-induced phophorylation of S6K1 (Thr421/Ser424) (Fig. 10C). In each condition, the peak response was maximal 10 min after stimulation.

Figure 10. 8-bromo-cGMP does not inhibit PE-induced phosphorylation of S6K1 (Thr421/Ser424).

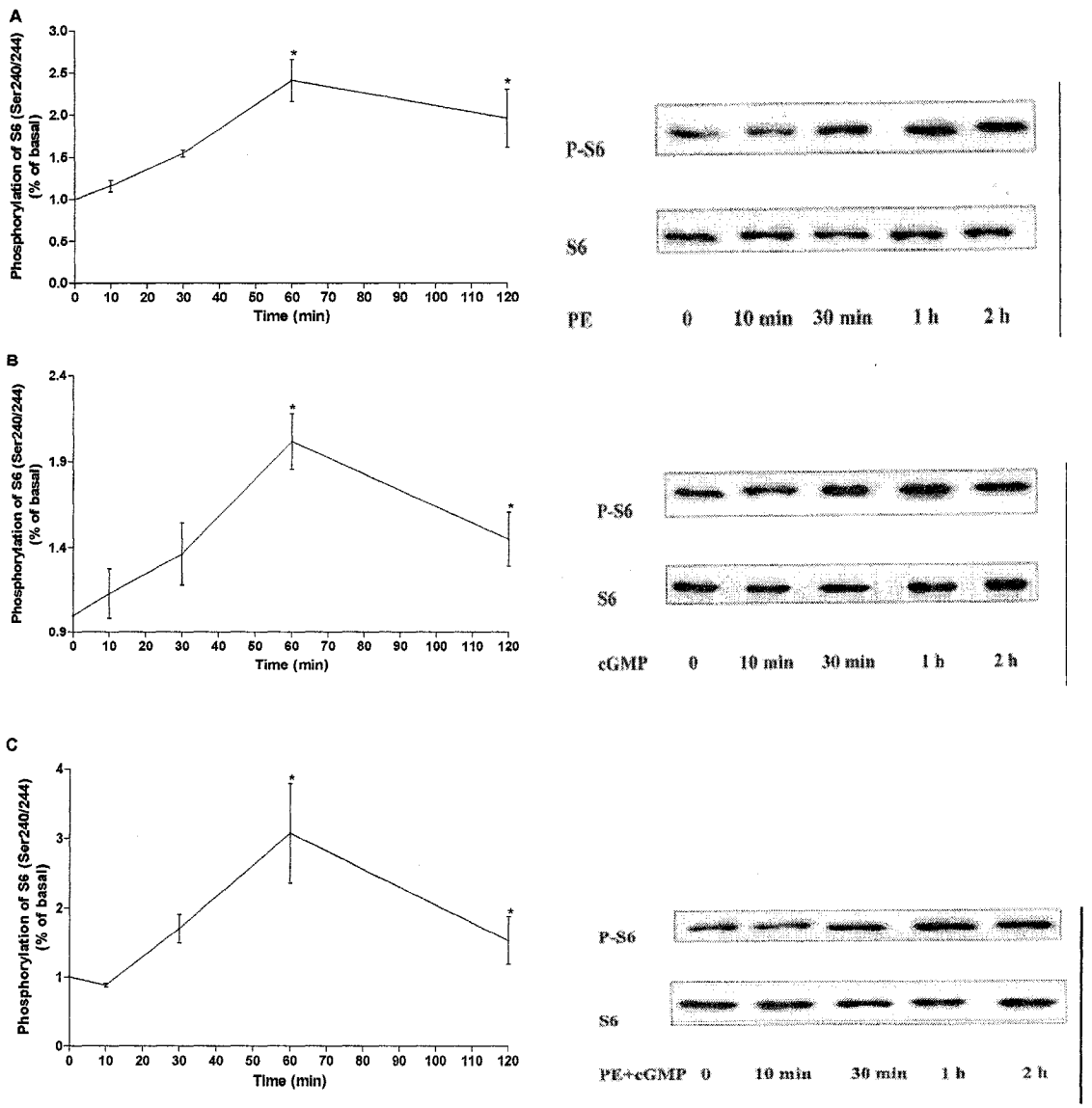


Cultured ARVC were treated with PE (10^{-5} M) (A), 8-bromo-cGMP (10^{-4} M) (B) and PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) (C) for the indicated times, and extracts were prepared as described under "methods". The phosphorylation of S6K1 (Thr421/Ser424) was assessed by Western blot analysis using a phosphor-specific antibody that recognizes S6K1 phosphorylated at the Thr421/Ser424. The antibody that detects S6K1 irrespective of its phosphorylation state was used to verify equal loading of the samples. Significant phosphorylation of S6K1 (Thr421/Ser424) by PE was observed during 10 min to 2 h stimulation and reached a maximum at 10 min ($p<0.05$, n=3). 8-bromo-cGMP alone also significantly phosphorylated S6K1 (Thr421/Ser424) ($p<0.05$, n=3) and the addition of 8-bromo-cGMP did not inhibit PE-induced phophorylation of S6K1 (Thr421/Ser424). All of the peak response of S6K1 (Thr421/Ser424) phosphorylaton by PE, 8-bromo-cGMP and PE + 8-bromo-cGMP were in 10 min, and still remained above baseline at 2 h in PE and PE + 8-bromo-cGMP groups. Data are means ± SD of three independent experiments. * $P<0.05$ *vs.* basal.

Phosphorylation of S6K1 is accompanied by later phosphorylation of ribosomal S6 protein

Ribosomal S6 protein is regulated and activated by S6K. To study whether PE-dependent phosphorylation of S6K1 does indeed lead to increased phosphorylation of rpS6, we treated ARVC with PE (10^{-5} M) for different times. Significant phosphorylation of ribosomal S6 (Ser240/244) was observed during the period ranging from 10 min to 2 h after stimulation, and reached a maximum at 1 h as assessed by Western blot analysis ($p<0.05$, n=3) (Fig. 11A). 8-bromo-cGMP (10^{-4} M) alone also significantly increased ribosomal S6 (Ser240/244) phosphorylation ($p<0.05$, n=3) (Fig. 11B). The addition of 8-bromo-cGMP did not inhibit PE-induced phophorylation of ribosomal S6 (Ser240/244) (Fig. 11C). In each condition, the peak response was maximal 1 h after stimulation.

Figure 11. Phosphorylation of S6K1 is accompanied by later phosphorylation of ribosomal S6 protein.

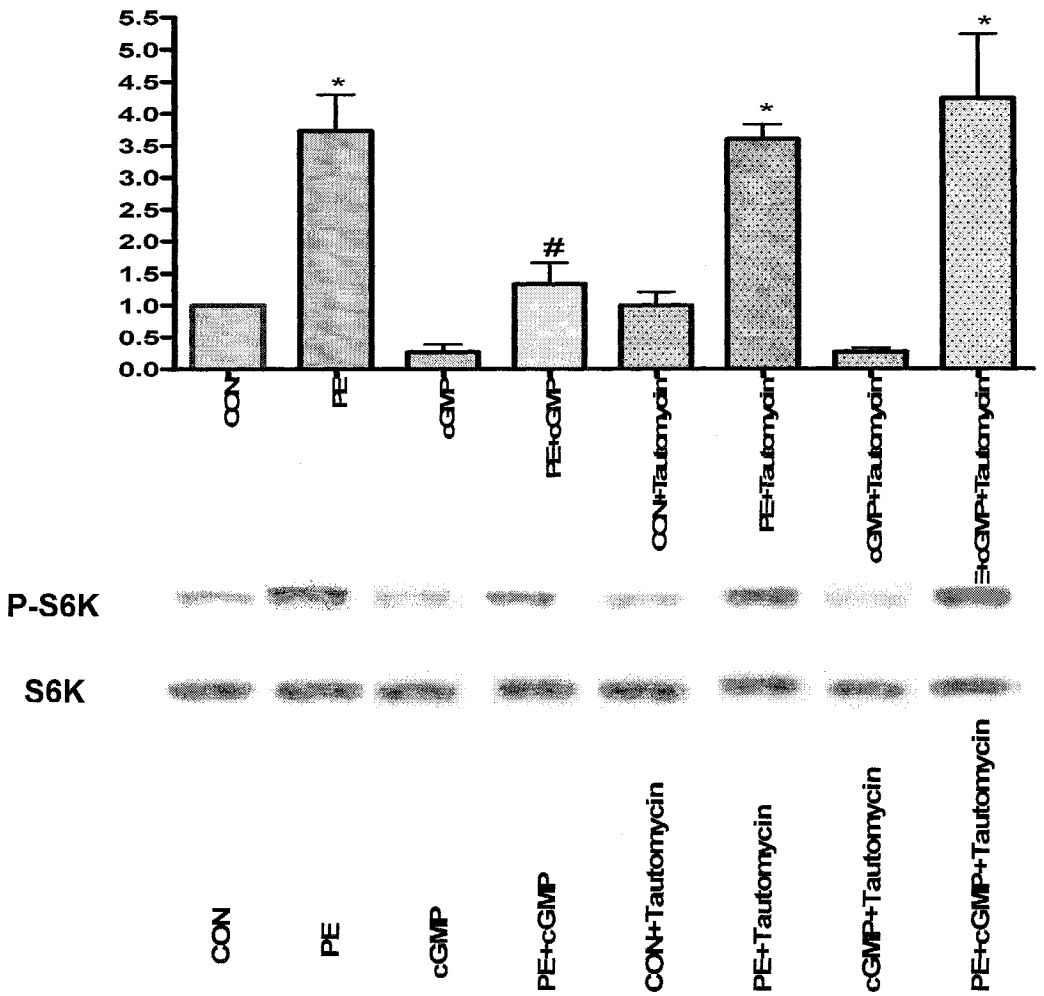


Cultured ARVC were treated with PE (10^{-5} M) (A), 8-bromo-cGMP (10^{-4} M) (B) and PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) (C) for the indicated times, and extracts were prepared as described under "methods". The phosphorylation of ribosomal S6 (Ser240/244) was assessed by Western blot analysis using a phosphor-specific antibody that recognizes ribosomal S6 protien phosphorylated at the Ser240/244. The antibody that detects ribosomal S6 irrespective of its phosphorylation state was used to verify equal loading of the samples. Significant phosphorylation of ribosomal S6 (Ser240/244) was observed during 10 min to 2 h stimulation and reached a maximum at 1 h ($p<0.05$, n=3). 8-bromo-cGMP alone also significantly phosphorylated ribosomal S6 (Ser240/244) ($p<0.05$, n=3), and the addition of 8-bromo-cGMP did not inhibit the peak response of PE-induced phosphorylation of ribosomal S6 (Ser240/244). All of the peak response of ribosomal S6 (Ser240/244) phosphorylaton by PE, 8-bromo-cGMP and PE + 8-bromo-cGMP were in 1 h. Data are means ± SD of three independent experiments. * $P<0.05$ *vs.* basal.

cGMP analog blocks phosphorylation of S6K1 by PE, but not phosphorylation of ribosomal S6 protein at 6 h treatment. Phosphatase inhibitor Tautomycin completely abolishes the inhibitory effect of cGMP analog on S6K1.

ARVC were incubated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) +8-bromo-cGMP (10^{-4} M) or media alone for 6 h. PE increased phosphorylation of S6K (Thr412/Ser424) ($p<0.05$, n=3), but 8-bromo-cGMP did not stimulate phosphorylation of S6K at 6 h. Moreover, 8-bromo-cGMP blocked phosphorylation of S6K induced by PE at 6 h treatment ($p<0.05$, n=3). Furthurmore, the phosphatase inhibitor Tautomycin, but not cyclosporine A (data not shown) completely abolished the inhibitory effect of 8-bromo-cGMP on S6K and this indicates that the cGMP analog activates phosphatases to inhibit phosphorylation of S6K (Fig. 12). PE also increased phophorylation of ribosomal S6 protein (Ser240/244). 8-bromo-cGMP did not stimulate phosphorylation of S6 at 6 h, and also did not inhibit phosphorylation of S6 by PE. Phosphatase inhibitors did not have an effect on phosphorylation of ribosomal S6 protein (data not shown).

Figure 12. 8-bromo-cGMP blocks phosphorylation of S6K1, but not ribosomal protein S6, induced by PE at 6 h treatment. Phosphatase inhibitor Tautomycin completely abolished the inhibitory effect of cGMP analog on S6K1.

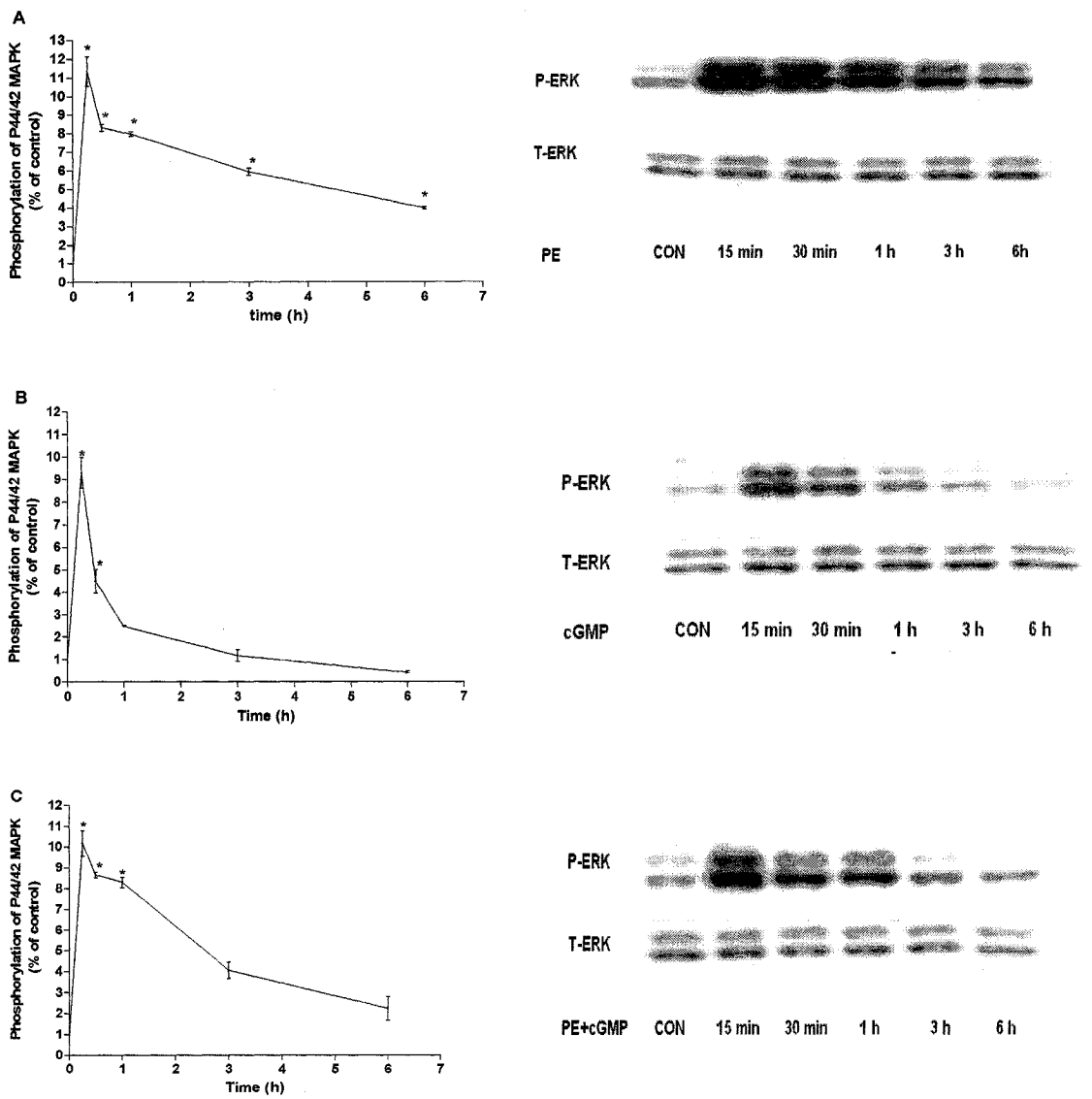


ARVC were incubated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) +8-bromo-cGMP (10^{-4} M) or media alone for 6 h. Extracts were prepared as described under "methods". The phosphorylation of S6K (Thr412/Ser424) was asessed by Western blot analysis using phospho-specific antibodies that recognize S6K1 phosphorylated at the Thr421/Ser424 and rpS6 phosphorylated at Ser240/244. The antibodies that detect S6K1 irrespective of their phosphorylation state were used to verify equal loading of the samples. PE increased phosphorylation of S6K1 ($p<0.05$, n=3), but 8-bromo-cGMP did not stimulate phosphorylation of S6K at 6 h. Moreover, 8-bromo-cGMP blocked phosphorylation of S6K induced by PE at 6 h treatment ($p<0.05$, n=3). Furthurmore, the phosphatase inhibitor Tautomycin, but not cyclosporine A (data not shown) completely abolished the inhibitory effect of 8-bromo-cGMP on S6K and this indicates that the cGMP analog activates phosphatases to inhibit phosphorylation of S6K. PE also increased phophorylation of ribosomal S6 protein (Ser240/244). 8-bromo-cGMP did not inhibit phosphorylation of S6 by PE at 6 h treatment. Phosphatase inhibitors did not have effect on phosphorylation of ribosomal S6 protein (Data not shown). Data are means ± SD of three independent experiments. * $p<0.05$ *vs.* con, # $p<0.05$ *vs.* PE

8-bromo-cGMP inhibits the phosphorylation of p44/42 MAPK (Thr202/Tyr204) by PE at long term treatment, but not at the short term treatment.

ERK is upstream of S6K1 and has been reported to be essential for PE-induced protein synthesis [93,192]. MEK inhibitor blocks increased S6K1 activity induced by PE[192]. However, it has also been suggested that selective activation of ERK is required for the antihypertrophic action of cGMP/PKG I[193]. To study the effect of PE and cGMP on ERK activity, we treated ARVC with PE (10^{-5} M), cGMP (10^{-5} M) and PE (10^{-5} M) + cGMP (10^{-4} M) or media alone for 15 min, 30 min, 1 h, 3 h and 6 h.. Significant phosphorylation of p44/42 MAPK (Thr 202/Tyr204) was observed during the period ranging from 15 min to 6 h after PE stimulation, and reached a maximum at 15 min as assessed by Western blot analysis ($p<0.05$) (Fig. 13A). 8-bromo-cGMP alone also significantly increased p44/42 MAPK phosphorylation ($p<0.05$), although phosphorylation appeared to return to baseline at 3 h after stimulation (Fig. 13B). The addition of 8-bromo-cGMP did not inhibit phosphorylation of p44/42 MAPK by PE during the period ranging from 15 min to 1 h, but inhibited PE-induced phosphorylation of p44/42 MAPK at 3 h and 6 h treatment (Fig. 13C). In each condition, the peak response was maximal 10 min after stimulation.

Figure 13. 8-bromo-cGMP inhibits the phosphorylation of p44/42 MAPK (Thr202/Tyr204) by PE at long term treatment, but not at the short term treatment.

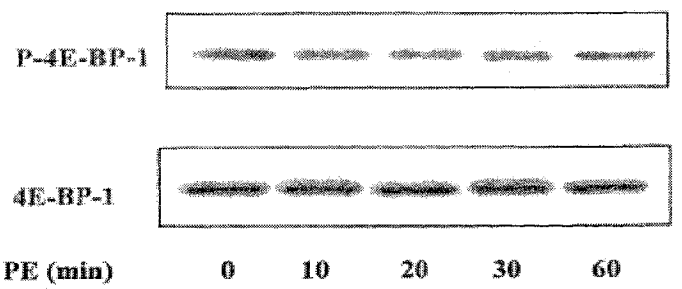


Cultrued ARVC were treated PE (10^{-5} M) (A), cGMP (10^{-5} M) (B) and PE (10^{-5} M) + cGMP (10^{-4} M) (C) or media alone for 15 min, 30 min, 1 h, 3 h and 6 h, and extracts were prepared as described under "method". The phosphorylation of p44/42 MAPK (Thr202/Tyr204) was assessed by Western blot analysis using a phosphor-specific antibody that recognizes p44/42 MAPK phosphorylated at the Thr202/Tyr204. The antibody that detects p44/42 MAPK irrespective of its phosphorylation state was used to verify equal loading of the samples. Significant phosphorylation of p44/42 MAPK (Thr 202/Tyr204) was observed during the period ranging from 15 min to 6 h after PE stimulation, and reached a maximum at 15 min as assessed by Western blot analysis ($p<0.05$). 8-bromo-cGMP alone also significantly increased p44/42 MAPK phosphorylation ($p<0.05$), although phosphorylation appeared to return to baseline at 3 h after stimulation. The addition of 8-bromo-cGMP did not inhibit phosphorylation of p44/42 MAPK by PE during the period ranging from 15 min to 1 h, but inhibited PE-induced phosphorylation of p44/42 MAPK at 3 h and 6 h treatment. In each condition, the peak response was maximal 10 min after stimulation. Data are means±SD of three independent experiments. * $P<0.05$ vs control.

4E-BP-1

4E-BP-1 is a key factor involved in regulating translation efficiency. Phosphorylated 4E-BP-1 dissociates from eIF4E, leading to the formation of eIF4F complex, which results in the initiation of translation[71]. We studied if PE phosphorylates 4E-BP-1. ARVC were treated with PE (10^{-5} M) for 10, 20, 30 and 60 min. Western blot was used for detecting the phosphorylation status of 4E-BP-1. In our culture conditions, we observed that 4E-BP1 was already phosphorylated even in the absence of other external stimulus. In unstimulated cells, 4E-BP-1 (Thr37/46) already phophorylated and there was no difference between basal and PE-treated groups (Fig. 14).

Figure 14. 4E-BP1



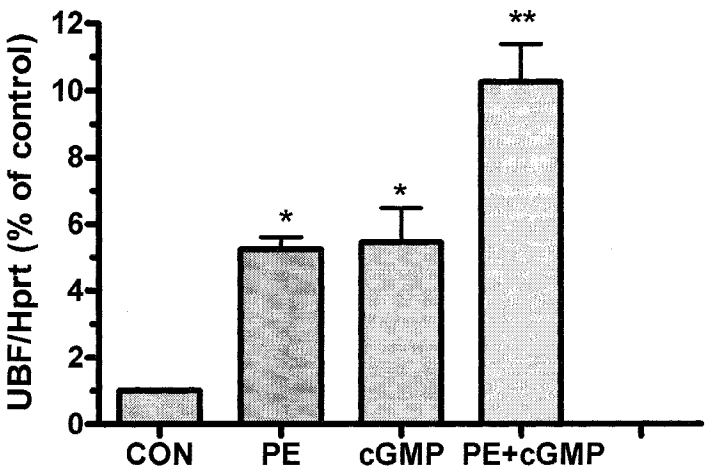
ARVC were treated with PE (10^{-5} M) for 10 min, 20 min 30 min and 60 min. Western blot was used for detecting phosphorylaiton status of 4E-BP-1. In unstimulated cells, 4E-BP-1 (Thr37/46) already phophorylated and there was no difference between basal and PE-treated groups.

The effect of cGMP analog on PE-induced increased UBF mRNA expression

UBF is strongly implicated in rDNA transcription, which determines in part protein translation capacity. Increased UBF content and phosphorylated UBF increase rDNA transcription, leading to increased translation capacity. We first wanted to determine whether PE phosphorylates the UBF protein, but we didn't have success in detecting UBF by Western blot using nuclear extracts. It is probably because UBF expression level is low in adult cardiomyocytes, as we found by Q-PCR analysis that UBF mRNA expression level is much lower than most of other genes (data not shown).

We then used Q-PCR to detect mRNA expression levels under PE stimulation and cGMP treatment in ARVC. ARVC were treated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 6 h. PE significantly increased UBF mRNA expression ($p<0.01$, $n=3$). Surprisingly, 8-bromo-cGMP also increased UBF mRNA expression to the same level ($p<0.01$, $n=3$) and addition of 8-bromo-cGMP with PE showed additive effects on increasing UBF mRNA expression ($p<0.001$, $n=3$) (Fig. 15).

Fingure 15. The effect of cGMP analog on PE-induced increased UBF mRNA expression



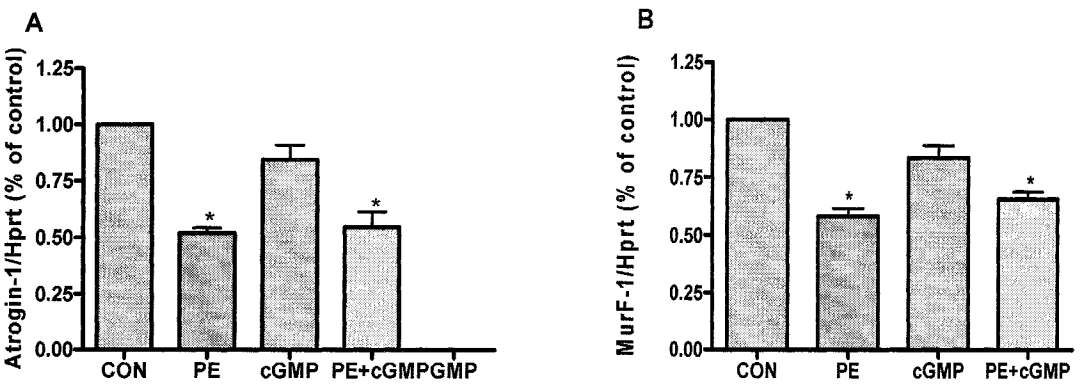
ARVC were treated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 6 h, and Q-PCR was performed to evaluate UBF mRNA expression level. Both PE and 8-bromo-cGMP increased UBF mRNA expression level ($P<0.01$, n=3) and the addition of 8-bromo-cGMP and PE induced additive effects on UBF mRNA expression level ($p<0.001$, n=3). Data are presented as means ± SD. * $p<0.01$ *vs.* con; ** $p<0.001$ *vs.* con

The effect of cGMP analog on PE-suppressed mRNA expression of Atrogin-1 and MurF-1

Uniquitin proteasome system (UPS) is the major proteolytic system in striated muscle cells[105]. Proteins are firstly ubiquitinated, and subsequently degraded by proteasomes. The ubiquitin ligases (E3) recognize a limited subset of target proteins and keep the specificity of UPS. Atrogin-1 and MurF-1 are two E3s that have received much attention in context of cardiac hypertrophy. It has been shown that overexpression of Atrogin-1 and MurF-1 inhibit cardiac hypertrophy[112,113]. Recent studies have suggested that the transcription factor FOXO proteins, which are negatively regulated by the PI3K/Akt pathway, increase the transcription of Atrogin-1 and MurF-1[115-117]. ARVC were treated

with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 3 h, Q-PCR was used for analyzing Atrogin-1 and MurF-1 mRNA expression. PE significantly decreased both Atrogin-1 (Fig. 16A) and MurF-1 (Fig. 16B) mRNA expression levels ($p<0.01$, *vs.* con n=4). 8-bromo-cGMP alone had no effect on Atrogin-1 and MurF-1 mRNA expression ($p>0.05$, *vs.* con, n=4), but addition of 8-bromo-cGMP with PE did not significantly increase mRNA expression level ($p>0.05$, *vs.* PE n=4).

Figure 16. The effect of cGMP analog on PE-suppressed mRNA expression of Atrogin-1 and MurF-1



ARVC were treated with PE (10^{-5} M), 8-bromo-cGMP (10^{-4} M), PE (10^{-5} M) + 8-bromo-cGMP (10^{-4} M) or media alone for 3 h, and Q-PCR was performed to evaluate Atrogin-1 and Murf-1 mRNA expression levels. PE significantly decreased both Atrogin-1 (A) and MurF-1 (B) mRNA expression ($p<0.01$, n=4). 8-bromo-cGMP alone had no effect on Atrogin-1 and MurF-1 mRNA expression ($p>0.05$, *vs.* con, n=4), but addition of 8-bromo-cGMP with PE did not significantly increase mRNA expression ($p>0.05$, *vs.* PE; $p<0.05$ *vs.* con n=4). Data are presented as means ± SD. * $p<0.01$ *vs.* con

SECTION 5: DISCUSSION

Increased protein accumulation is a key feature of cardiac hypertrophy, which constitutes one of the most powerful risk factors for cardiovascular mobility and mortality[194]. cGMP has recently emerged as an endogenous regulator for controlling or reversing cardiac hypertrophy[195,196]. Numerous *in vitro* or *in vivo* studies have confirmed that cGMP/PKG mediates the antihypertrophic effects of natriuretic peptides, NO and bradykinin[195,196]. 8-bromo-cGMP is a cell-permeable cGMP analog which is more resistant to phosphodiesterases than cGMP and preferentially activates PKG. It has been demonstrated that 8-bromo-cGMP inhibits increased protein accumulation under prehypertrophic stimuli[132-134,142], but the molecular mechanisms responsible for this effect have not been elucidated yet. S6K1 is a well-known positive regulator of protein synthesis, and acts by increasing the translation of mRNAs that encode for components of the protein synthetic machinery during different hypertrophic stimuli[197,198]. In the present study, we have confirmed that 8-bromo-cGMP inhibits PE-stimulated accumulation of newly synthesized proteins in cultured ARVC. Both of PE and 8-bromo-cGMP increase phosphorylation of S6K1 in short time stimulation (10 min to 2 h), but 8-bromo-cGMP blocks S6K1 phosphorylation induced by PE at 6 hour treatment. Furthermore, our data suggest that 8-bromo-cGMP blocks S6K phosphorylation by activating phophotases, as treatment with the phosphatase inhibitor, Tautomycin, abolishes the inhibitory effect of 8-bromo-cGMP on S6K phosphorylation. We then show that cGMP induces transient increased phosphorylation of ERK and inhibits PE-increased phosphorylation of ERK at longer time treatment (3 h and 6 h). We have also investigated the effects of 8-bromo-

cGMP on regulation of protein degradation. 8-bromo-cGMP does not have effect on the PE-induced downregulation of the Atrogin-1 and MurF-1 mRNA transcripts. We have also extended this observation by establishing that 8-bromo-cGMP inhibits PE-induced generation of ROS. Altogether, our study provides the first evidence that the cGMP analog inhibits PE-induced protein acculumation in ARVC via activation of phosphatases that prevents the long-term phosphorylation of S6K1.

It has been well documented that ANP, BNP and NO antagonize cardiac hypertrophy via cGMP/PKG signaling pathway in vitro and in vivo [130-136,141-145]. A large body of evidence has also accumulated to show that ANP, BNP and NO inhibit protein synthesis by α-adrenergic receptor agonists and Ang II in cultured cardiomyocytes [132,133,142,184]. Furthermore, 8-bromo-cGMP analogs and an inhibitor of cGMP specific phosphodiesterase suppress PE-induced increase in protein synthesis [132,134,142,143]. We used cultured ARVC to confirm that 8-bromo-cGMP inhibits PE-induced increase in protein synthesis evaluated by [^{3}H] phenylalanine incorporation (Fig. 8B).

In general, cGMP effectors include PKG (PKG I and PKG II), cGMP-gated cation channels and cGMP-regulated phosphodiesterases[199]. In cardiomyocytes, PKG I appears to be the predominant isoform and mediates antihypertrophic effects of cGMP [159]. Upregulation of PKG I inhibits protein synthesis induced by α-adrenoreceptor stimulation, while inhibition of PKG I enhances the cardiac hypertrophy induced by a-adrenoreceptor agonist [142,160]. However, the antihypertrophic mechanisms of cGMP/PKG I are still unclear. Some studies have reported that cGMP/PKG I exerts antihypertrophic

actions by inhibiting Ca^{+}-calcineurin-NFAT signaling, Rho A/Rho kinase, MAPK signaling, NHE, TnI or downregulating MLP, but the mechanism by which cGMP/PKG I regulates signaling pathways involved in protein synthesis has not been elucidated yet[155,160,169,171,173-175,200].

S6K1 has been well recognized as an important positive regulator of protein synthesis. It is widely accepted that activated S6K1 increases translation of mRNA which encode exclusively for components of the translation machinery, including all ribosomal proteins, elongation factors, and poly(A)-binding protein (PABP)[201,202]. Different hypertrophic stimuli activate S6K1 in cardiomyocytes and overexpression of S6K1 induces cardiac hypertrophy [94-96]; while $S6K1^{-/-}$ mice display smaller cell size of all organs [202]. Due to the critical role of S6K1 in protein synthesis, we hypothesized that cGMP may exert the inhibitory effect on protein synthesis induced by PE via inhibition of S6K1 activity. To address this central question, we have assessed the effect of 8-bromo-cGMP on S6K1 phosphorylation by western blot analysis. PE increases phosphorylation of S6K1 during the period of 10 min to 2 hour stimulation, which is in agreement with previous studies[192,203] (Fig. 10A). However, 8-bromo-cGMP alone also increases phosphorylation of S6K1 and does not block PE-increased phosphorylation of S6K1 in short time treatment (10 min to 2 h) (Fig. 10 B and C). In contract, 8-bromo-cGMP blocks phosphorylation of S6K1 at longer time treatment (6 h) (Fig. 12 A). It has been shown that PKC, ERK and mTOR all lay upstream of the activation of S6K1 [192,203,204]. cGMP might activate phosphatases to dephosphorylate the upstream kinases of S6K1 or S6K1 directly, resulting in the inhibition of S6K1. Therefore we have also examined the effect of phosphatase inhibitors on S6K1. We have treated cardiomyocytes with phosphatase inhibitors cyclosporine A (an inhibitor of calcineurin) and tautomycin (an inhibitor of PP1

and PP2A). Tautomycin completely abolishes the inhibitory effect of 8-bromo-cGMP on S6K1 phosphorylation induced by PE (Fig. 12); In contrast, cyclosporine A does not show any effect on phosphorylation of S6K 1 at 6 h treatment (Data not shown). Since tautomycin inhibits PP1 and PP2A, it appears likely that 8-bromo-cGMP activates phophatases, which then dephosphorylate either PKC, MAPK, mTOR or S6K1 itself.

We also looked at the effects of 8-bromo-cGMP on phosphorylation of ERK and ribosomal S6 protein, which lay upstream and downstream of S6K1, respectively.

At present, there are nine S6K substrates described, in which rpS6, mTOR, S6K1 Aly/REF-like target (SKAR), eIF4B, eEF2K/eEF2 are involved in controlling protein synthesis[100,155,160,169,171,173-175,200]. rpS6 is the first one described and it has received the most attention. Both of PE and 8-bromo-cGMP increase phosphorylation of rpS6, and 8-bromo-cGMP does not inhibit PE-increased phosphorylation of rpS6 during the period of 10 min to 2 h stimulation (Fig. 11) and at 6 h. rpS6 had been implicated in the regulation of 5'-TOP mRNA, which encode for proteins that is part of the translational machinery. It had first been suggested that phosphorylation of rpS6 by S6K1 increases the affinity of ribosomes for 5'-TOP mRNA, resulting in an increase rate of translational initiation. But other investigators have reported that phosphorylation of rpS6 does not result in enhanced protein synthesis in Ehrlich cells [97]. It also has been shown that the growth of S6-difficient hepatocytes is not affected[205]. The discrepancies between these previous studies have recently been resolved by the finding that the translation control of 5'-TOP mRNA is not affected in rpS6 $^{p-/-}$ mice. In fact, global protein synthesis rate is higher in mouse embryo fibroblasts from rpS6$^{p-/-}$ mice compared to wide type. Moreover, their findings further indicated that the proportion of ribosomes engaged in polysomes is similar in wild

type and rpS6 $^{p-/-}$ mice [206]. S6K1 might be a fine-tuning mechanism to balance protein synthesis with minimum energy wastage by which activation of S6K1 upregulates protein synthesis by activating eEF2 kinase and/or eIF4B and concomitantly downregulates protein synthesis by phosphorylation of rpS6. Phosphorylated rpS6 might also compete with other S6K substrates, resulting in hypophosphorylation and inactivation of other substrates to inhibit protein synthesis[100]. This might explain why 8-bromo-cGMP blocks PE-increased phosphorylation of S6K1, but that rpS6 remains phosphorylated during the same time period.

We have also examined ERK, which lies upstream of S6K1. Activated ERK was previously thought to be a critical signaling event for initiating the hypertrophic response in PE stimulation [192,207,208]. However, other evidences have shown that ERK inhibition fails to down-regulate biochemical markers of hypertrophy in PE-stimulated cardiomyocytes [209], and even enhances skeletal muscle cell differentiation in IGF-1 treated cells [210]. Furthermore, Silberbach et al. have shown that PE, ANP and 8-bromo-cGMP increases phosphorylation of ERK in neonatal cardiomyocytes. Interestingly their study also shows that the MEK inhibitor PD098059 completely abolishes ANF-induced inhibition of PE-induced hypertrophy, which suggests that selective activation of ERK (not p38 MAPK and c-JUN) is required for the antihypertrophic action of cGMP/PKG I[193]. In our study, PE and 8-bromo-cGMP alone induce sustained (during the period ranging from 15 min to 6 h) and transient (during the period of 15 min to 1 h) increased phosphorylation of ERK, respectively. The addition of 8-bromo-cGMP does not inhibit PE-induced phoshorylation of ERK at short times, but inhibits PE-induced phosphorylation of ERK at later times (3 h and 6 h) (Fig. 13). The ERK activity duration has been considered as a critical factor for cellular responses[211]. It has been shown that

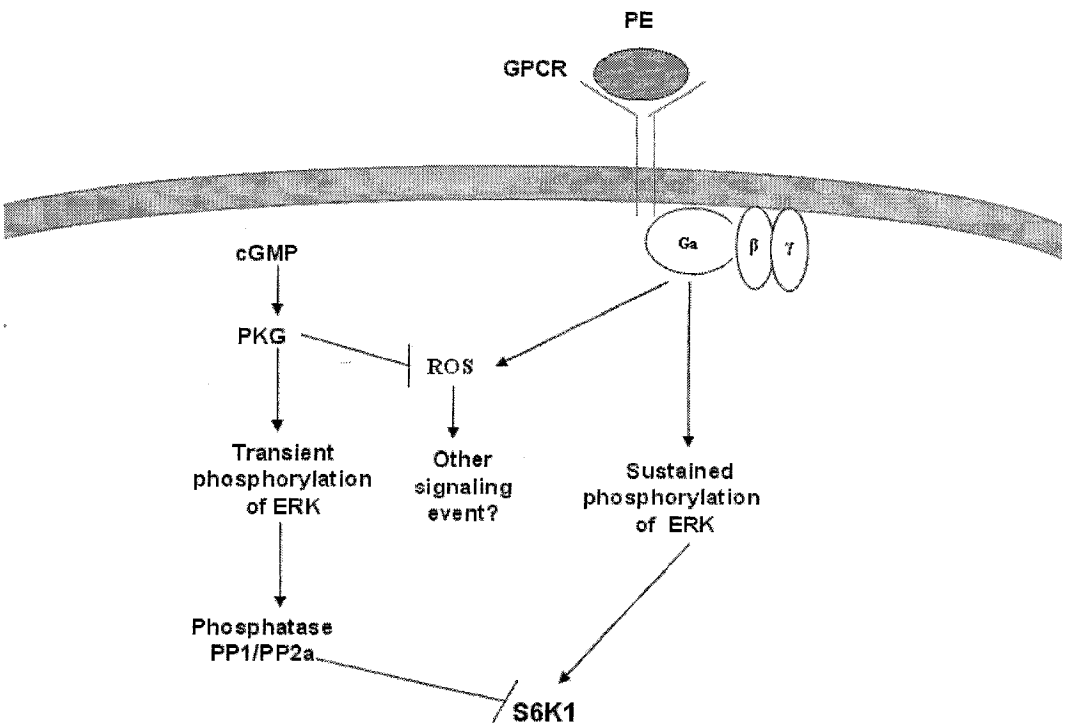
sustained, but not transient activation of ERK induces quiescent fibroblasts to proliferate[211,212]. Moreover, only sustained ERK activation phosphorylates and stabilizes proteins that are encoded by early genes such as *Jun* and *Fos*, and these early gene encoded proteins function as transcription factors and might change gene expression level crucial for cell proliferation. [213,214]. We presume that transient phosphorylation of ERK by cGMP activates phosphatases to inhibit S6K1, and the inhibitory effect of cGMP on PE-induced phosphorylation of ERK also directly exert negative regulation on protein synthesis.

It has been shown that ANP activates MAPK-phosphatase-3 (MKP-3) in human vascular smooth muscle cells; likewise L-Arginine increases MKP-1 protein expression, which is inhibited by PKG inhibitor in cardiomyocytes [172,215]. Other studies have also shown that both 8-pCPT-cGMP and 8-bromo-cGMP induce MPK-1 mRNA expression in different cell types. It is worth noting that PKG I-dependent induction of MKP-1 mRNA expression occurs between 30 min and 1 h of drug exposure [173,216,217]. It has also been shown that the activation of ERK signaling cascasdes induces MKP-1 expression [218]. So it might be that the transient activation of ERK by cGMP induces MKP-1 expression, and then in turn increased MKP-1 inhinit ERK activation at later time.

In our study, 8-bromo-cGMP rapidly stimulates ERK and S6K1, but blocks PE-induced phosphorylation of S6K1 and ERK at later times. cGMP might phosphorylate S6K1 via ERK activation at short time, and then in turn activate ERK-inducible phosphatases to dephosphorylate the kinases involved in positive controlling of protein synthesis including S6K1. This would be consistent with the observation that 8-bromo-cGMP

phosphorylates S6K1 in 2 h treatment and subsequently blocks phosphoryltion of S6K1 at 6 h treatment. The inhibitory effect of cGMP on ERK at later time also participates in the negative regulation of protein synthesis. Fig. 17 summarizes the mechanism of cGMP to inhibit PE-increased phosphorylation of S6K.

Figure 17. The mechanism of cGMP to inhibit PE-increased phophorylation of S6K1



PE stimulates GPCR to induce sustained phosphorylation of ERK, leading to phosphphorylation of S6K1. cGMP activates PKG in cardiomyocytes, and this induces transient phosphorylation of ERK and then in turn activate phosphatase (PP1/PP2A) to dephosphorylate S6K1. cGMP also inhibit ROS generation induced by PE to exert antihypertrophic effect.

4E-BP1 is a substrate of S6K1 and regulates the in initiation of translation. However, in our culture condition, 4E-BP1 was already phosphorylated in the absence of exogenous stimulation, and was not further phosphorylated by PE (Fig. 14). This is consistent with

another previous study which has shown that 4E-BP1 exists in a predominantly phosphorylated state in unstimulated cardiomyocytes [219].

Increased UBF content and phosphorylation increase rDNA transcription, leading to increased translation capacity. It has been shown that increased UBF expression during α-adrenoceptor stimulation is required for hypertrophic growth in neonatal cardiac myocytes [78]. PE stimulates UBF mRNA expression, but 8-bromo-cGMP fails to inhibit this effect, and surprisingly leads to additional increase in UBF mRNA expression (Fig. 15). However it noteworthy that almost all of the studies on UBF were performed on neonatal cardiomyocytes, and the rates of rDNA transcription in nuclei isolated from adult cardiomyocytes is about one-fifth of rates in nuclei from neonatal cardiomyocytes[220]. The present study was performed using adult cardiomyocytes, and we found that UBF is expressed at low level in adult cardiomyocytes. Indeed UBF protein cannot be detected by western blot analysis, and the UBF cDNA level is much lower compared to other genes (such as Hypoxanthine-guanine phosphoribosyltransferase and myofibrillogenesis-regulator-1) by Q-PCR method. UBF might not be the critical regulator of rDNA transcription in adult cardiomyocytes.

During the development of cardiac hypertrophy, both protein synthesis and protein degradation are activated[221]. However, the increase rate of protein synthesis is higher than protein degradation, leading to the net increase in protein accumulation[4]. UPS are an important proteolytic system for protein degradation in cardiomyocytes. However, there are some discrepancies concerning the role of protein degradation during cardiac

hypertrophy. Some studies suggest that hypertrophic stimuli increase protein synthesis by Akt/mTOR pathway, and concomitantly decrease protein degradation by Akt/FOXO pathway[106]. It also has been shown that proteasome activities are depressed during cardiac hypertrophy. However, other investigators suggest that protein degradation also increased with increased proteasome activity during development of cardiac hypertrophy[118,221]. The controversy may be due to the different roles of UPS during different phases of development of hypertrophy and different experimental models. Atrogin-1 and MurF-1 are E3s involved in ubiquitination of substrate proteins and have received much attention in the context of cardiac hypertrophy. It has been suggested that Atrogin-1 and MurF-1 target calcineurin and TnI for proteasome-dependent degradation, respectively; likewise, both of them attenuate cardiac hypertrophy in vitro[112-114]. We have shown that PE inhibits Atrogin-1 and MurF-1 mRNA expression, and 8-bromo-cGMP does not exert these effects (Fig. 16). It has been demonstrated that Atrogin-1 and MurF-1 are increased by FOXO transcription factor, which is negatively regulated by Akt. This suggests that cGMP might not inhibit Akt signaling. Since it has been demonstrated that proteasome activities are increased during chronic pressure overload and proteasome inhibitors completely prevent cardiac hypertrophy [118]. In the future, we will examine the effect of PE and cGMP analog on proteasome activities in ARVC to explore the role of UPS during PE-stimulated protein accumulation and the effect of cGMP analog on protein degradation.

Moreover, we have demonstrated that 8-bromo-cGMP inhibits ROS generation induced by PE (Fig 9), compatible with a recent study showing that inhibition of NOS (and thus presumably of intracellular cGMP) induces both increased protein synthesis and a rapid increase in ROS formation[201]. ROS are involved in cardiac hypertrophy by activating

multiple signaling pathways such as MAPK, PKC, Rho A and NHE [50,60-62]. By reducing ROS generation, cGMP inhibits the kinase cascades involved in protein accumulation, and might also contribute to inactivation of S6K1.

In summary, our study dissects out the molecular mechanism by which cGMP analog inhibits increased protein accumulation induced by PE. In particular, cGMP analog limits the time during which S6K1 is phosphorylated after PE stimulation, presumably via the induction of tautomycin-sensitive phosphatases.

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SECTION 7: APPENDIX

ad 05

CONTEX ENVIRONNEMENT

certifie que **Ying Li**
a complété la formation décrite au verso

PRINCIPES DE RADIOPROTECTION

CONTEX ENVIRONNEMENT

certifie que **Hamid Massarli**
a complété la formation décrite au verso

PRINCIPES DE RADIOPROTECTION