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Research Article

Calcitonin Gene-Related Peptide Regulates Cardiomyocyte Survival through Regulation of Oxidative Stress by PI3K/Akt and MAPK Signaling Pathways

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Abstract

CGRP and specific CGRP receptors are found in the heart where they produce positive-inotropic and anti-apoptotic effects, key adaptations to exercise and cardiovascular disease. PI3K/Akt and MAPK signaling imbalances are associated with cardiomyocyte pathologies; however, the effects of CGRP on these pathways are unclear. Therefore, we hypothesized that CGRP modulates inotropic and apoptotic adaptations of cardiomyocytes by regulating PI3K/Akt and MAPK/ERK signaling balances. We treated cardiomyocytes with combinations of CGRP, PI3K/Akt and MAPK signaling agonists and antagonists. We evaluated expression of the mRNA and proteins levels of survival signaling molecules related to the PI3K/Akt and MAPK and measured apoptosis by caspase 3/7 activity. $CGRP_{1-37}$ decreased Akt, NFκB, SOD-3 and increased ERK1/2 and p38 MAPK expressions, which was antagonized by $CGRP_{8-37}$. Akt-negative construct transfection, Ad.Akt(K179M), inhibited the $CGRP_{1-37}$-induced increment in MAPK expressions. A PI3K-antagonist treatment with LY294002 or $CGRP_{1-37}$/Ad.Akt(K179M) co-treatment alleviated the CGRP-increased caspase activity and -decrements in SOD-3.

These findings demonstrate a CGRP negative effect on the PI3K/Akt signaling pathway and CGRP receptor-induced crosstalk between PI3K/Akt and MAPK in normal cardiomyocytes. Future studies to differentiate CGRP effects on intracellular signal transduction mechanisms in pathological conditions will elucidate the significance of CGRP in, and provide novel therapeutic targets for, heart failure.

INTRODUCTION

Activity of CGRP and specific CGRP receptors in the heart produce positive-inotropic [1,2] and anti-apoptotic [3,4] effects, which are key adaptations to exercise and cardiovascular disease. CGRP is a 37-aminoacid, regulatory peptide derived by alternative splicing of the calcitonin gene located on chromosome 11 and one of a family of multifunctional peptides that includes amylin and adrenomedullin (AM) [5]. Amylin is also a 37-aminoacid peptide, named for its deposition of amyloid and role in glycemic control, released from the pancreas with insulin. Amylin inhibits gastric motility and appetite, thereby regulating blood glucose [6-8]. AM is a 52-aminoacid peptide, named for the pheochromocytoma cell in which it was originally discovered, is highly expressed in cardiac and vascular tissues and, like CGRP, is a potent vasodilator [9]. CGRP is also synthesized in and released from sensory neurons, a mediator of pain signaling and plays a central role in sensitizing the trigeminal ganglion to Ca^{2+} in migraine headache [10,11]. AM has both positive- and a negative-inotropic effects in cardiac myocytes [12], decreases papillary muscle contractile force (Bell et al 2010) and increases cell resistance to oxidative stress and production of NO [13]; whereas, CGRP increases cardiomyocyte contractile force [1] and is released by K^+ induction of Ca^{2+} currents [14] as well as by NO [15] and the pro-inflammatory cytokine TNF-α [16]. These calcitonin regulatory peptides appear to regulate Ca^{2+} fluxes, activate adenylate cyclase and, therefore, increase cellular cAMP activity [17,18] but by actions on different receptor motifs.

The two forms of CGRP are α-CGRP and β-CGRP are different by three aminoacids; however, β-CGRP is expressed from a separate gene that does not produce calcitonin [5,19-21]. Activity of CGRP depends on the calcitonin receptor-like receptor (CL), associated with G proteins, and three distinct receptor activity modifying proteins (RAMP1, RAMP2 and RAMP3). These RAMPs are determinants of membrane localization and binding specificity of CL receptors. A CL-RAMP1 complex constitutes the CGRP-1 receptor, activated by α-CGRP and CL-RAMP2 and CL-RAMP3 complexes are receptors for AM [22]. Although the nonfunctional $CGRP_{8-37}$ molecule antagonizes the CGRP-1 receptor, CGRP also binds to the CGRP-2 receptor that is not affected by $CGRP_{8-37}$ [23].

Abnormal plasma levels of AM and CGRP are reported in pre-eclampsia and other cardiovascular diseases associated with endothelial dysfunction [24]. Moreover, both AM and

CGRP appear to mediate positive-inotropy in cardiac myocytes [1,12]. These findings suggest that CGRP receptors could provide novel, specific targets for preventing and treating cardiovascular disease. Moreover, AM is reported to exert its effects by MAPK/ERK [25] and CGRP by PI3K/Akt intracellular signaling pathways, shared by other regulators of positive-inotropy [1,2]. There is also substantial crosstalk between these pathways in experimental models [4]. The early signs of cardiovascular disease include hypertension with increased contractile force and Ca^{2+} fluxes, leading to cardiac remodeling, fueled by oxidative stress with apoptosis [26]. However, the linkages between CGRP receptors and intracellular signal transduction pathways for positive-inotropy and anti-apoptosis remain unclear [27]. The present study was, therefore, designed to determine the relationships between specific CGRP-1 receptors and PI3K/Akt and MAPK/ERK pathways for signaling positive-inotropic and anti-apoptotic effects in cardiomyocytes.

MATERIALS AND METHODS

Conformity statement

All the procedures used in this study conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH) publication No. 85-23, revised 1996. The animal protocol has been independently approved by Howard University institutional animal care and usage committee.

Animal preparation

Male Sprague-Dawley rats, 200-250 g body weight, were purchased from Harlan Laboratories (Madison, WI). The animals were allowed to recover and become familiar with their new environment upon arrival to the animal house of the Howard University College of Medicine, for 1 week. The animals were housed in secure, clean and environmentally-controlled room temperature (70°F-74°F) with a 6:00 h to 18:00 h light cycle and were fed food and water *ad libitum*.

Tissue samples and treatment conditions

Cardiac tissue was obtained from adult male Sprague Dawley rats. Hearts were removed from the Sprague Dawley anesthetized rats (halothane) and perfused with either 10 μM CGRP1-37, 10 μM CGRP8-37, 1 μM LY249002; or adenovirus strain with a modified construct: Ad:Akt(K179M) or Ad:myrAkt alone or in combination in a perfusion buffer (11.9 mM NaCl, 46.9 mM KCl, 9.4 mM $MgSO_4$, 12.2 mM KH_2PO_4, 1 mM Ascorbic acid, 250 mM $NaHCO_3$, 115.4 mM Glucose, and 1 mM $CaCl_2$) for 45 min. The Ad:Akt(K179M) and Ad:MyrAkt both are adenoviral construct that expresses a kinase-inactive, dominant negative Akt mutant. The CGRP and LY249002 concentrations used were similar to previously reported effects of these molecules in the rodent cardiovascular system [1,28-31].

qRT-PCR

Total mRNA (from perfused heart tissue) was isolated using the Aurum Total RNA Fatty and Fibrous tissue Kit (Biorad; Hercules, CA) according to the manufacturer's manual. 1 μg of total mRNA was then used for reverse transcription and amplification using the SuperScript-III One-step RT-PCR kit (Life Technologies; Grand Island, NY) following the manufacturer's protocol. PCR was performed using Akt, SOD-3, NFκB, ERK1, and p38 MAPK primers. Rat beta-actin forward 5'-TCGTGCGTGACATTAAGGAG-3' and reverse 5'-GTCAGGCAGCTCGTAGCTCT-3'; endogenous rat sod3 forward 5'-GACCTGGAG ATCTGG ATGGA-3' and reverse 5'-GTGGTTGGAGGTGTTCTGCT-3'; AKT-1 forward 5'- CTGGGTTACCCCGGTGTGT-3' and reverse 5'- GCACATCCGAGAAACAAAA-3'; ERK1 forward 5'- GAGCCCAGGGGAACTGCT-3' and reverse 5'-CTGGAAGCGGGCTGTCTC-3'; P38/MAPK14 forward 5'- AGGAGAGGCCCACGTTCTAC-3' and reverse 5'- TCAGGCTCTTCCATTCGTCT-3'. β-actin was employed as an internal control. The Biorad iQ5 cycler was used for the qRT-PCR.

Caspase 3/7 activity assay

Caspase 3/7 activity has been measured according to the manufacturer instructions, Promega (WI). Accordingly, protein extracts from treated homogenized cardiac tissue were incubated for 30 minutes in 96-well plates. Caspase-3/7 activity reagent (Promega, WI) was added to samples in 1:1 dilutions. This reagent causes lysis of the cell and cleavage of the DEVD-aminoluciferin substrate, which is freed and degraded by luciferase enzyme. Thus, a luminescent signal is emitted corresponding to caspase-3/7 activity. The samples were analyzed using Victor V^3 multiplate plate reader (Perkin Elmer) at the excitation wavelength of 485 nm.

Western blotting assay

Total protein was isolated from rat hearts and exposed to RIPA lysis buffer which was composed of: EGTA(1 mM),) EDTA (2mM), DTT (2 mM), benzamidine (10 mM), b-glycerophosphate (20 mM), Na_3VO_4 (0.2 mM), NaF (20 mM), $NaVO_3$ (0.5 mM), 0.6% deoxycholate, 0.1% Triton X-100, and 1 tablet/10 mL of complete protease inhibitors. The lysates were incubated on ice for 15 min and centrifuged for 20 min at a speed of 14,000 rpm. Protein concentrations were recorded from the samples, separated by SDS-PAGE and transferred onto nitrocellulose membranes where NFκB, ERK1/2, phospho-ERK1/2, p38 MAPK, GAPDH (as control) antibody probes were used to display protein expression. The above mentioned probes along with the secondary anti-rabbit monoclonal antibody were employed in this protocol (Cell signaling). Bands were visualized by chemiluminescence. Membranes from three separate experiments were scanned and the densities of the bands were evaluated using the NIH "Image J" software package.

Statistical methods

Statistical analyses were performed using Prism 6.0 (Graphpad) software and verified using Microsoft Excel, which gave the same results. Paired Student's t-tests were used to compare the pre- and post-treatment data for the same animal group. The heteroscedastic two-sample unpaired Student's t-test, assuming unequal variances, was used to compare treatment effects between two different animal groups. Using the null hypothesis, $P \leq 0.05$ was significant.

RESULTS

Effects of CGRP on the survival and the proliferative pathways gene expression

We initially evaluated the direct effects of CGRP on Akt gene expression in hearts perfused with $CGRP_{1-37}$ alone and

in combination with the PI3K/Akt activator (IGF-1) or the PI3K inhibitor LY294002. We also incorporated an adenoviral construct containing coding for kinase-inactive dominant negative Akt mutant in cardiac tissue using Ad.MyrAkt or Ad.Akt(K179M). As shown in (Figure 1A), the $CGRP_{1-37}$ treatment decreased Akt mRNA expression (-1.48 ± 0.36 fold, P<0.05). Inhibition of PI3K or transfection with Ad.MyrAkt also decreased Akt mRNA expression in the presence of $CGRP_{1-37}$ (-1.78 ± 0.67 fold and -1.20 ± 0.84 fold, respectively, P<0.05). The IGF-1 treatment increased Akt mRNA expression (1.41 ± 1.08 fold, P=0.03), even in the presence of $CGRP_{1-37}$, thereby counteracting the effects of $CGRP_{1-37}$. As expected from an acute effect, changes in gene expression are small but significant. We also evaluated the effects $CGRP_{1-37}$ on NFκB mRNA expression, downstream of Akt. (Figure 1B) shows that the $CGRP_{1-37}$ treatment decreased NFκB mRNA expression (-3.36 ± 0.81 fold, P<0.05). This CGRP1-37-induced decrement in NFκB mRNA expression was blocked by LY294002 and by Ad.Akt(K179M) treatments (1.70 ± 0.66 fold, P<0.05 as compared to $CGRP_{1-37}$ alone).

The effects of $CGRP_{1-37}$ on SOD-3 mRNA expression are shown in (Figure 1C). The $CGRP_{1-37}$ treatment decreased SOD-3 mRNA expression (-2.03 ± 0.68 fold, P<0.05). To evaluate the associations of $CGRP_{1-37}$, Akt and SOD activities, we treated hearts with $CGRP_{1-37}$ in combination with either Ad.Akt(K179M), or LY294002. These co-treatments decreased the $CGRP_{1-37}$-induced decrement in SOD-3 mRNA expression (-0.675± 0.99 fold, P=0.04 and -1.16 ± 0.78 fold, P=0.02 compared to $CGRP_{1-37}$ alone, respectively). The IGF-1 treatment also decreased the $CGRP_{1-37}$- induced decrement in SOD-3 mRNA expression.

Parallel MAPK signaling molecules such as ERK1 and p38 MAPK are shown to respond to stress stimuli associated with apoptosis, growth factors, interleukins, and interferons. Therefore, we evaluated the effects of CGRP on mRNA expression of these MAPKs. As shown in (Figure 1D), the $CGRP_{1-37}$ treatment increased ERK1 mRNA expression (1.54 ± 0.80 fold, P<0.05). This $CGRP_{1-37}$-induced increment in ERK1 mRNA expression was effectively antagonized by co-treatment using the dominant-negative Ad.Akt(K179M) (-2.88 ± 1.00 fold, P=0.007). The $CGRP_{1-37}$ and IGF-1 co-treatment failed to further modulate the $CGRP_{1-37}$-induced increment in ERK1 mRNA expression. The $CGRP_{1-37}$ treatment decreased p38 MAPK mRNA expression as depicted in (Figure 1E) (-2.14 ± 0.32 fold, P<0.05). The $CGRP_{1-37}$ co-treatments using LY204002 and Ad.Akt(K179M) failed to further modulate the $CGRP_{1-37}$-induced decrement in p38 MAPK mRNA expression. The IGF-1 treatment decreased the $CGRP_{1-37}$-induced decrement in p38 MAPK mRNA expression (-0.90 ± 0.38 fold, P=0.02).

Effects of CGRP on survival and proliferative pathways activities

Recently, we have shown that $CGRP_{1-37}$ treatment similar to that used in this study decreased the expression of Akt protein [1]. This $CGRP_{1-37}$ induced decrement in Akt expression was

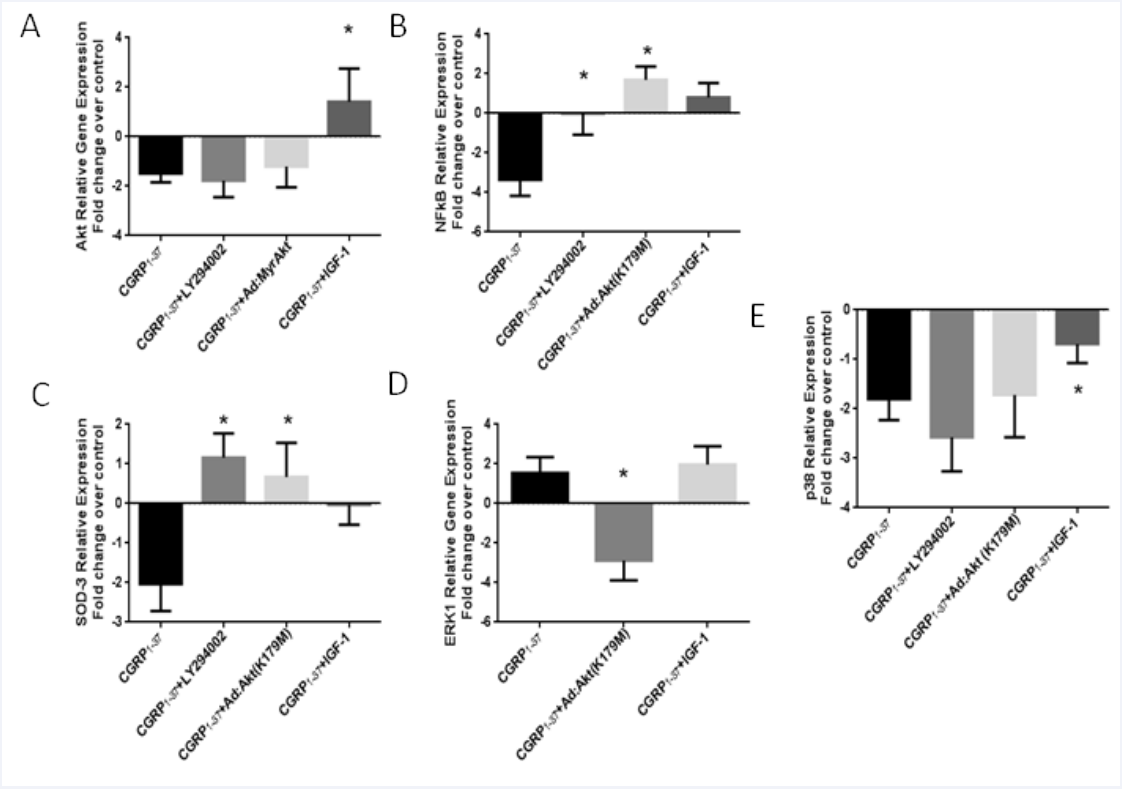

Figure 1 **Effects of CGRP on the survival and the proliferative pathways gene expression. A.** The $CGRP_{1-37}$ + IGF-1 treatment increased Akt mRNA expression. All other treatments decreased Akt mRNA expression. **B.** The $CGRP_{1-37}$ + LY294002 and the $CGRP_{1-37}$ + Ad.Akt(K179M) treatments both increased SOD-3 mRNA expression, **C.** All the treatments decreased p38 MAPK mRNA expression. **D.** The $CGRP_{1-37}$ treatment decreased NFκB mRNA expression $CGRP_{1-37}$. NFκB expression was increased by the $CGRP_{1-37}$ + LY294002, the $CGRP_{1-37}$ + IGF-1 and the $CGRP_{1-37}$ + Ad.Akt(K179M) treatments. **E.** The $CGRP_{1-37}$ + Ad.Akt(K179M) treatment decrease ERK1 mRNA expression. (N=6, * P<0.05).

effectively antagonized by the $CGRP_{8-37}$ treatment. IGF-1 co-treatment also decreased the $CGRP_{1-37}$-induced decrement in Akt protein expression. In this study, the $CGRP_{1-37}$ treatment increased ERK1 protein activity (56.02 ± 14.15%, P=0.03 compared to control). This $CGRP_{1-37}$-induced increment in ERK1 activity was antagonized by the $CGRP_{8-37}$ or by the Ad.Akt(K179M) co-treatments (Figure 2). IGF-1 co-treatment did not further affect the $CGRP_{1-37}$-induced increment in ERK1 activity (77.88 ± 6.09%, P=0.02 compared to control). There was no significant effect of $CGRP_{1-37}$ on ERK2 protein activity.

Figure 3A shows that the $CGRP_{1-37}$ treatment increased p38 MAPK protein expression (75.72 ± 1.62%). The $CGRP_{1-37}$-induced increment in p38 MAPK expression was effectively antagonized by the $CGRP_{8-37}$ or Ad.Akt(K179M) co-treatments, but not by the LY294002 co-treatment. The co-treatment with IGF-1 reduced but did not alleviate the $CGRP_{1-37}$ effect on p38 MAPK protein expression (46.48 ± 3.86%; P=0.05 compared to control; which is -56.42 ± 1.16%, P=0.05 compared to $CGRP_{1-37}$ alone). (Figure 3B) shows that the $CGRP_{1-37}$ treatment did not change NFκB protein expression significantly (-8.02 ± 0.34%, P>0.1). The LY294002 or the Ad.Akt(K179M) co-treatments decreased NFκB protein expression (-14.77 ± 0.36% and -19.67 ± 0.46%, P=0.05). The IGF-1 co-treatment increased NFκB protein expression marginally (9.95 ± 0.60%, P= 0.10).

Effects of $CGRP_{1-37}$ on cellular apoptosis

Figure 4 demonstrates that the $CGRP_{1-37}$ treatment and the $CGRP_{1-37}$/IGF-1 co-treatment had no direct effects on caspase 3/7 activity. In order to verify the functionality or the responsiveness of the caspase 3/7 in our preparation, we inhibited the PI3K/Akt pathway with LY294002 treatment which increased caspase 3/7 activity (27.0 ± 11.2%, P=0.02) and with $CGRP_{1-37}$/Ad.Akt(K179M) co-treatment which also increased caspase 3/7 activity (19.2 ± 1.7%, P=0.02).

DISCUSSION

The main finding of this study is that physiologically-active $CGRP_{1-37}$ treatments shifted the intracellular signaling balance in normal cardiomyocytes. These effects of $CGRP_{1-37}$ were, largely, antagonized by pretreatments with the physiologically-inactive specific CGRP-1 receptor blocker $CGRP_{8-37}$ which decreased specific activities of PI3K/Akt cell survival signal transduction molecules and increased specific activities of MAPK/ERK, oxidative stress and apoptosis transduction molecules. The effects of $CGRP_{1-37}$ not antagonized by $CGRP_{8-37}$ suggest that some of the effects of CGRP were mediated by the CGRP-2 receptor.

In this study we have shown that the $CGRP_{1-37}$ has a detrimental effect on the survival signaling pathway related to PI3K/Akt in the heart. To that effect we have demonstrated that $CGRP_{1-37}$ induces a reduction in Akt gene expression that corroborates with a lower Akt protein activation level. This effect seems to be a direct effect of $CGRP_{1-37}$ as transfection with the dominant negative Akt or inhibition of its direct upstream effector, PI3K, induced the same level of decrement in Akt gene expression similar to what we have recently found with its protein activity level [1]. Interestingly, IGF-1 offsets the $CGRP_{1-37}$ effect, which may indicates that Akt is sufficient and necessary

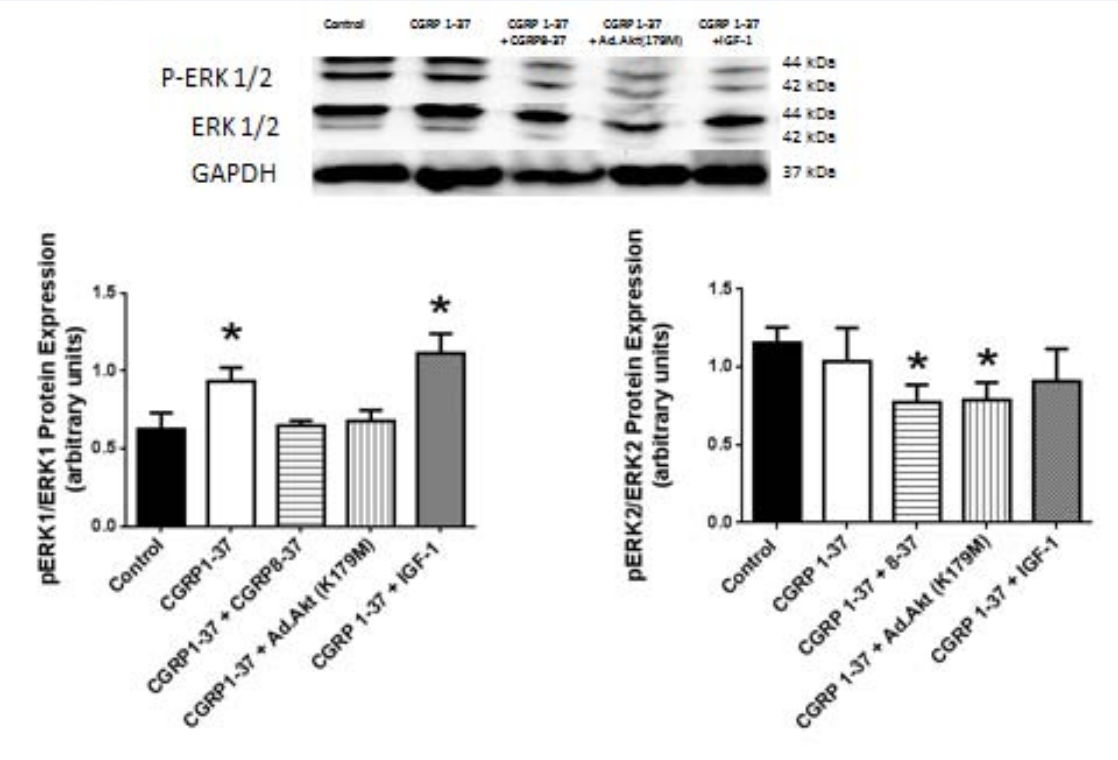

Figure 2 Effects of CGRP on the proliferative pathways activities, ERK1 and ERK2. Upper: sample western blots for ERK1 (left) and ERK2 (right) total and phosphorylated form with the corresponding loading control GAPDH. Lower: Bar graphs depicted the effects of $CGRP_{1-37}$ in the presence or absence of antagonists ($CGRP_{8-37}$) or Akt activator- (IGF-1) or down-regulator (Ad.Akt(K179M)). (N= 6, * p<0.05).

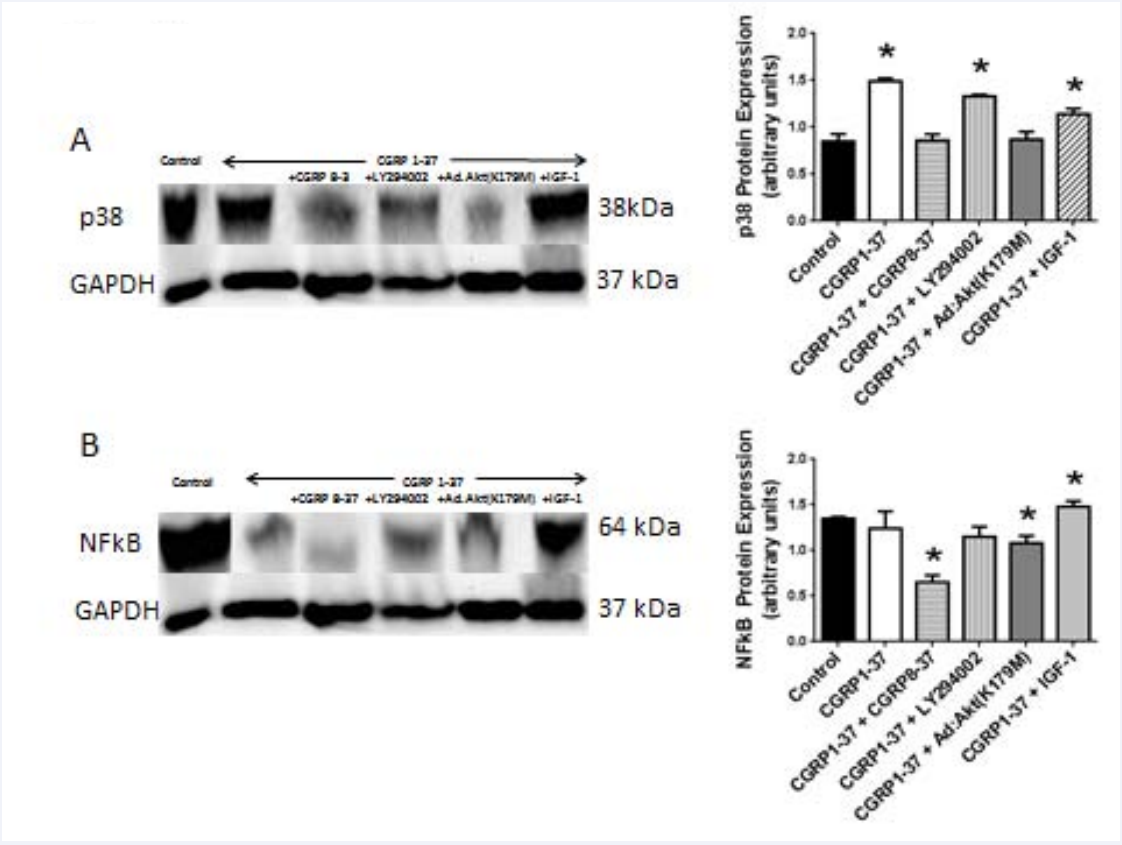

Figure 3 Effects of CGRP on the survival pathways activity, p38 MAPK and NFkB. A. The $CGRP_{1-37}$, the $CGRP_{1-37}$ + LY294002 and the $CGRP_{1-37}$ + IGF-1 treatments increased p38 MAPK protein expression. **B.** $CGRP_{1-37}$ decreased NFκB protein expression and the $CGRP_{1-37}$ + IGF-1 treatment increased NFκB protein expression (N=6, * P<0.05).

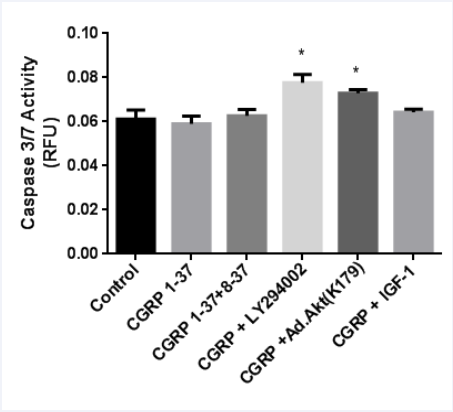

Figure 4 Effects of CGRP1-37 on cellular apoptosis. Caspase 3/7 activity from protein extracts that were treated alone with $CGRP_{1-37}$ or in combination with $CGRP_{8-37}$, LY294002, Ad.Akt (K179M) and IGF-1. Caspase activity is expressed as relative fluorescence units (RFU). The $CGRP_{1-37}$ treatment did not directly affect caspase 3/7 activity. The $CGRP_{1-37}$ + LY294002 and $CGRP_{1-37}$ + Ad.Akt(K179M) treatments increased caspase 3/7 activity (P<0.05). The IGF-1 + $CGRP_{1-37}$ treatment had no effect on caspase 3/7 activity (N=3; * P<0.05).

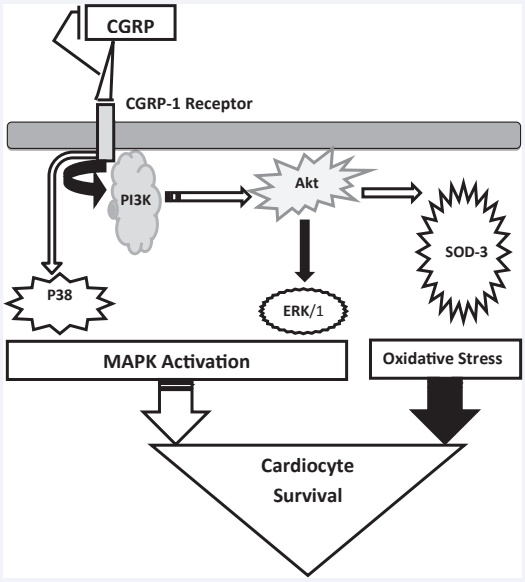

Figure 5 Diagram showing the inhibitory effect of CGRP on the PI3K/Akt pathway protein expression via the CGRP-1 receptor. P38-MAPK and ERK1/2-MAPK are activated by $CGRP_{1-37}$ in an Akt-independent and dependent manner, respectively. The activation levels of the latter versus the level of oxidative stress may dictate the overall cellular response to $CGRP_{1-37}$. Filled arrows designate an inhibitory action, while unfilled arrows denote an activation process.

signaling switch for the CGRP effects. It has been recently shown that nerve growth factor (NGF) improves neurite outgrowth [32,33] mainly through PI3K/Akt activation of cGMP in CGRP-containing DRG neurons [34,35]. Furthermore, NGF is reported

to induces expression of CGRP in DRG [36]. Thus, in accordance with our present data, it seems likely that the CGRP is part of a regulatory mechanism that monitors the NGF activation of the PI3K/Akt signaling pathway. No comparable studies have yet been performed on cardiac myocytes which makes the present report novel and significant. Akt signaling is central to many cellular survival mechanisms and decreased Akt expression or activation could, therefore, be a key factor in a number of pathophysiological events and sequelae [37]. Accordingly, the present study demonstrates that the $CGRP_{1-37}$ treatment also decreased mRNA expressions of the anti-oxidant enzyme SOD-3, as well as the anti-apoptotic Akt-downstream effector, NFκB. We realize that changes in mRNA levels are limited, but this is expected from a short-term acute effect. These anti-survival effects were produced by CGRP-induced down-regulation of Akt because they were prevented by either PI3K inhibition or dominant-negative Akt. The PI3K/Akt signaling agonist IGF-1 also counteracted the CGRP-induced decrements in NFκB and SOD3 mRNA expression, thereby corroborating the central role of Akt. This finding is also consistent with previously reported CGRP effects on cell survival and cardiac inotropic function [1]. Nonetheless, this finding contradicts a previous report that CGRP alleviated SOD activity in a model of hyperoxia-induced lung injury [38]. However, our findings agree with those of others demonstrating that exogenous CGRP decreased NFκB and induced apoptosis in thymocytes [39]. These disparate findings suggest tissue-specificity in the downstream apoptotic and/or oxidative effects of CGRP. Thus, the finding that the $CGRP_{1-37}$ treatment decreased both Akt and NFκB mRNA expression, the latter downstream of Akt mRNA expression, suggests that such NFκB mRNA expression is indicative of the capacity for CGRP to employ the entire PI3K/Akt cell survival signaling pathway that includes an anti-apoptotic effect. This interpretation is bolstered by the finding that LY294002 and Ad.Akt(K179M) decreased basal NFKB mRNA expression.

In cardiac myocytes, we previously demonstrated cross reactivity, also called crosstalk, between the PI3K/Akt and MAPK signaling pathways [4]. The MAPK pathway, particularly ERK1 and p38 MAPK, has been implicated in signaling cellular proliferation such as that which occurs in the development of cardiac hypertrophy, [28,29,40,41]. Thus, it was important to probe such interactions in the context of the $CGRP_{1-37}$ deactivation of the Akt activity. We found that the $CGRP_{1-37}$ treatment increased ERK1 mRNA and protein expression in an Akt-dependent manner. This was evidenced by the finding that $CGRP_{1-37}$-induced increase in ERK1 mRNA expression was inhibited by dominant-negative Akt co-transfection. These findings may suggest that CGRP modulates ERK1 partly via Akt signaling. Similar findings are reported in hepatocytes and PC12 cells, suggesting PI3K positive crosstalk with ERK1/2 [42,43]. On the other hand, the $CGRP_{1-37}$ treatment noticeably reduced p38 MAPK mRNA expression, independently of PI3K/Akt, but enhanced p38 MAPK protein expression in an Akt-dependent manner. This peculiar interaction suggests an auto-regulatory translational mechanism involving Akt, whereby a CGRP induced reduction in Akt activation may have relieved an Akt-driven inhibition of p38 MAPK protein synthesis, perhaps by an epigenetic mechanism. This was evidenced by the findings that PI3K inhibition (which decreases Akt activation) mimicked the $CGRP_{1-37}$ treatment effect on p38 MAPK and that the $CGRP_{1-37}$ and IGF-1 co-treatment significantly dampened the $CGRP_{1-37}$-enhanced p38 MAPK protein expression. The fact that the Ad.Akt(K179M) co-transfection blocked this $CGRP_{1-37}$-induced p38 MAPK effect suggests that Akt activation rather than the Akt protein expression level is relevant here. An epigenetic hypothesis for exogenous CGRP signaling is also consistent with the finding that although the $CGRP_{1-37}$ treatment decreased NFκB mRNA expression, it had no effect on NFκB protein expression. Therefore, it is suggestive that IGF-1 has the capacity to counteract the CGRP-induced decrement in p38 MAPK mRNA expression.

All these $CGRP_{1-37}$ induced effects on PI3K/Akt, MAPK and NFκB were blocked by the calcitonin receptor-like receptor (CALCRL) antagonist $CGRP_{8-37}$ thereby indicating that $CGRP_{1-37}$ was acting via its membrane receptor on the cardiomyocytes. These results imply that $CGRP_{1-37}$ weakens the anti-apoptotic and strengthens the proliferative signaling pathways, notably in an Akt-dependent manner.

The cellular biomarkers for apoptosis, caspase 3/7 activity were apparently not modulated directly by exogenous CGRP. However, inhibition of Akt by either the LY294002 or the Ad:Akt(K179M) treatment increased the caspase 3/7 activity, irrespective of $CGRP_{1-37}$, indicating responsiveness of the caspases to changes in Akt expression. Thus, it seems that enhancement of signaling in a pathway parallel to PI3K/Akt, the MAPK/ERK pathway, may have counter-balanced the decrement in anti-apoptotic signaling via the PI3K/Akt pathway. A compensatory activation of ERK1 induced by down-regulation of PI3K/Akt signaling is reported in transgenic mice [44]. To the extent that, as we describe herein, MAPK/ERK signaling enhancement can be Akt-dependent, Akt appears to be playing an auto-regulatory role in maintaining cell survival in the presence of $CGRP_{1-37}$.

In summary, this is the first study to demonstrate the effects of CGRP on the PI3K/Akt and the MAPK pathways for cell survival, apoptosis and stress. As depicted in the diagram in (Figure 5), on one hand CGRP induces down-regulation of the PI3K/Akt/SOD pathway which may lead to elevated oxidative stress. On the other hand, this CGRP effect does not affect NFκB nor caspase 3/7 activity, which could be due to the observed enhancement of the anti-apoptotic MAPK (ERK1/2 and p38) pathways [45]. Furthermore, in our setting ERK1/2 activation seems to be Akt dependent, whereas p38 is mostly Akt-independent. P38-MAPK is known to respond to environmental stress such as the oxidative ones induced by CGRP [45]. Thus, the activation levels of the both MAPKs versus the level of oxidative stress may dictate the overall cellular response to CGRP. These effects of CGRP treatments demonstrate that the PI3K/Akt cell survival and MAPK cell anti-apoptotic (ERK) and stress (p38) signaling pathways are not exclusive, exhibiting substantial interdependence, connectivity and crosstalk. These findings together with those of previous studies from our laboratory, showing $CGRP_{1-37}$-induced positive-inotropic effects correlated with changes in Ca^{2+} fluxes in cardiomyocyte, sarcomere and whole heart preparations; suggest that CGRP receptors could be useful targets for preventing and treating cardiovascular disease. Future studies to differentiate the effects of CGRP on the intracellular signal transduction mechanisms in pathological

conditions, such as cardiac hypertrophy will, no doubt, help elucidate the significance of CGRP dysregulation in, and provide novel therapeutic targets for, heart failure.

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