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Calcitonin gene-related peptide exerts inhibitory effects on autophagy in the heart of mice

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ABSTRACT

Calcitonin Gene-Related Peptide (CGRP) is a potent vasodilator peptide widely distributed in the central nervous system and various peripheral tissues, including cardiac muscle. However, its role in heart protein metabolism remains unknown. We examined the acute effects of CGRP on autophagy and the related signaling pathways in the heart mice and cultured neonatal cardiomyocytes. CGRP (100 μg kg^{-1}; s.c.) or 0.9 % saline was injected in awake male C57B16 mice, and the metabolic profile was determined up to 60 min. In fed mice, CGRP drastically increased glycemia and reduced insulinemia, an effect that was accompanied by reduced cardiac phosphorylation levels of Akt at Ser^{473} without affecting FoxO. Despite these catabolic effects, CGRP acutely inhibited autophagy as estimated by the decrease in LC3II:LC3I and autophagic flux. In addition, the fasting-induced autophagic flux in mice hearts was entirely abrogated by one single injection of CGRP. In parallel, CGRP stimulated PKA/CREB and mTORC1 signaling and increased the phosphorylation of Unc51-like kinase-1 (ULK1), an essential protein in autophagy initiation. Similar effects were observed in cardiomyocytes, in which CGRP also inhibited autophagic flux and stimulated Akt and FoxO phosphorylation. These findings suggest that CGRP in vivo acutely suppresses autophagy in the heart of fed and fasted mice, most likely through the activation of PKA/mTORC1 signaling but independent of Akt.

1. Introduction

The calcitonin gene-related peptide (CGRP) is a neuropeptide composed of a sequence of 37 amino acids formed from the alternative splicing of the calcitonin gene [1]. It is widely distributed in the nervous and cardiovascular systems [2]. CGRP is synthesized and secreted by sensory nerve fibers of type C and Aδ [3], where it is frequently colocalized with the substance P [4]. Once released, CGRP binds to its calcitonin receptor-like (CLR), which belongs to the G protein family [5]. The binding of CGRP to its receptor activates the signaling pathway of the cAMP (cyclic adenosine monophosphate) [2], which in turn activates the classic effector PKA (cAMP-dependent protein kinase) that phosphorylates various proteins such as CREB (cAMP-responsive element-binding protein) and the hormone-sensitive lipase (HSL) [6].

The presence of sensory nerve fibers positive to CGRP in the heart and around peripheral arteries [7] suggests a potential physiological role of this neuropeptide in the heart under basal and stressful conditions. Indeed in the heart, CGRP causes coronary vasodilation, increases the heart rate and the force of contraction [8]. Previous studies have shown that ischemia-reperfusion injury activates apoptosis and autophagy in the heart, and the activation of these proteolytic pathways might contribute to adverse cardiovascular function. On the other side, several studies have shown that CGRP exerts a cardiac protective action against ischemia and heart injury in experimental ischemia-reperfusion injury models [9,10]. Interestingly, previous studies have shown that CGRP also induces cardiac hypertrophy [11,12], suggesting a potential role of this peptide in the control of heart metabolism. However, this hypothesis has not been tested so far. In line with this notion, our laboratory recently demonstrated that CGRP inhibits muscle protein degradation and autophagy in either normal or atrophic muscles

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induced by motor neuron denervation [13,14].

Autophagy refers to a cellular catabolic process in which damaged, dysfunctional or harmful organelles or cytoplasmic proteins are transported to the lysosome to be thus degraded [15,16]. This maintenance of the degradation of cellular components is essential for the growth, development, and function of normal cells [17]. The autophagic process begins with two complexes activation: ULK1 (Unc51-like kinase 1) complexed with Atg13 (Autophagy-related protein 13) and FIP200 (Family interactin protein of 200 kD) and Beclin1 complexed with PI3K-III (phosphatidylinositol 3-kinase class III) and p150. LC3 (microtubule-associated protein light chain 3) is a protein that, when converted into its LC3-II isoform, assists in the elongation of the phagophore membrane and remains attached to it until its degradation by the lysosome. Thus, LC3-II is an essential marker of autophagic activity [18].

Posttranslational and transcriptional mechanisms can regulate the autophagic process. The posttranslational control of autophagy is mainly mediated by the activation of mTOR (mammalian target of rapamycin), a Ser/Thr protein kinase involved in the control of protein synthesis and autophagy [16]. The posttranslational control of autophagy-mediated by mTOR is mainly dependent on its inhibitory action on ULK1 Ser^{757} residue phosphorylation [19,20]. The transcriptional control of autophagy is mainly mediated by FoxO (Forkhead box class O) family members, transcriptional factors involved in regulating genes that encode autophagic proteins [21]. It is well established that insulin, a hormone secreted by beta-pancreatic cells, negatively controls the autophagic/lysosomal proteolytic system by activating mTOR or Akt, Ser/Thr protein kinase involved in the phosphorylation and inhibition of all FoxO family members [22,23]. Recent studies from our laboratory have shown that the cAMP/PKA signaling pathway activators such as catecholamines [24] and CGRP [13,14] negatively control autophagy in skeletal muscle. Moreover, we have also shown that PKA inhibits FoxO activity and regulates skeletal muscle plasticity [25]. Therefore, we hypothesize that CGRP inhibits autophagy in the heart of mice by recruiting the canonical signaling pathway of cAMP/PKA.

2. Material and methods

2.1. Experimental model

Male C57/BL6 mice (8–10 weeks of age, ~25 g) were maintained under a 12 h light/dark cycle with free access to water and food. All experiments and protocols followed the ethical principles adopted by the Brazilian College of Animal Experimentation and approved by Ribeirão Preto Medical School of the University of São Paulo-The Ethics Committee on Animal Use (CEUA 018/2010).

For measurements of metabolites and hormones, animals received one (s.c.) injection of saline (NaCl 0.9 %) (Control) or CGRP (BACHEM) (100 μg kg^{-1}) and were sacrificed after 15 (CGRP 15 min), 30 (CGRP 30 min), and 60 min (CGRP 60 min). The blood and left ventricle of the heart were collected for biochemical analyses.

Proteins and genes were analyzed in the heart of fed mice 15 and 60 min following one injection of CGRP (100 μg kg^{-1}), respectively. This short time of treatment has been chosen because of the the-short half-life of the peptide (~10 min) [26]. A separate group of animals was treated with one (i.p.) injection of $CGRP_{8-37}$ (100 μg kg^{-1}) (Sigma–Aldrich), a selective antagonist, or saline (NaCl 0.9 %) and sacrificed after 45 min.

2.2. Autophagic flux quantification

The autophagic flux was monitored in fed and fasted animals using a modified colchicine protocol (COL; C9754, Sigma-Aldrich) [27]. Animals were deprived of food or not for 24 h to analyze the autophagic flux. Briefly, mice were treated with one (i.p.) injection of vehicle (saline) or 0.4 mg kg^{-1} of colchicine. The treatment was administered three times, at 48, 24, and 1 h before the injection of CGRP or saline. Animals were sacrificed 15 min after the injection of CGRP or saline.

2.3. Primary cultures of neonatal rat cardiomyocytes

The hearts of neonatal (1–3 days old) Wistar rats were excised, and the ventricles were minced and transferred to a sterile buffer. The tissue was subjected to 6–7 subsequent enzymatic digestions with collagenase, each performed at 37 °C for 12 min. The solution obtained from each digest was then transferred to a tube containing 1 mL of newborn calf serum (NCS) and centrifuged. Each cell pellet was resuspended in NCS, and dissociated cells were pooled. To separate myocytes from non-myocytes, the cell suspension was layered onto discontinuous Percoll density gradients consisting of 2 phases. After washing to remove all traces of Percoll, the myocytes were cultured in DMEM containing 5% fetal calf serum, penicillin and streptomycin (P/S, 1 %), and 10 % horse serum for 48 h [28]. The next day, a group of cardiomyocytes was cultured in glucose with or without chloroquine (40μM), and during the last 15 min, cardiomyocytes were incubated in the presence or absence of CGRP (1μM).

2.4. Metabolites and hormone measurements

Blood glucose concentrations were determined using the glucometer Accu-Check Performa Nano (Roche). Serum insulin levels were measured using a method based on a competitive enzyme immunoassay system (Millipore). A commercial kit was used to measure the plasma concentration of free fatty acids (NEFA-RANDOX, UK). The indirect assessment of sympathetic activity was performed by measuring the content of noradrenaline in the heart and catecholamines in the plasma of animals using a method previously standardized in our laboratory [29].

2.5. Western blot analysis

Cardiac muscle tissues and cardiomyocytes were lysed and Western blot was performed previously [30]. Primary antibodies were detected using peroxidase-conjugated secondary antibodies (1:5000 for β-actin and 1:1000 for the other primary antibodies) and visualized using ECL reagents by an ImageQuant 350 detection system (GE Healthcare, Piscataway, NJ). Band intensities were quantified using ImageJ (version 1.43 u, National Institutes of Health, USA). Protein expression were corrected by expression of β-actin. The phosphorylated forms were corrected for the total forms. Cell Signaling primary antibodies Beclin1 (1:1000; #3495 P), LC3 (1:1000; 2775S); phospho-Ser^{473} Akt (1:1000; #9271); phospho-Thr^{308} Akt (1:500; #9275); phospho-Ser^{133} CREB (1:500, #9198 L); phospho-Ser^{256} FoxO1 (1:750; #9461); phosphor-Ser^{193} FoxO4 (1:1000, #9471S) phospho-Ser^{660} HSL (1:1000, 4126S) phospho-Ser^{2448} mTOR (1:500,29715); phosphor-Ser/Thr PKA substrates (1:1000,#9624S); phospho-$Ser^{235/236}$ S6 (1:1000; #2211S) phospho-Ser^{757} ULK1 (1:500, #6888S). Santa Cruz Biotechnology primary antibodies β-actin (1:1000; sc-81178). PROGEN Biotechnik primary antibodies p62 (1:750; gp62-e).

2.6. Quantitative PCR

Total RNA was prepared from mouse cardiac muscle and cardiomyocytes using TRIzol (Invitrogen, CA) kit. Complementary DNA (cDNAs) was generated with SuperScript IV reverse transcriptase (Invitrogen, CA). Real-time PCR was carried out using an ABI7500 sequence detection system (Applied Biosystems, CA). The cDNAs were subsequently submitted to real-time PCR using Platinum® SYBR® Green qPCR Supermix UDG (Invitrogen, CA) with specific primer sequences mice Sik1 (f-tccaccaccaaatctcaccg; r-gtttcggcgctgcctcttc), Map1lc3b (f-cgtcctggacaagaccaagt; r-attgctgtcccgaatgtctc), CathepsinL (f-gtggactgttctcaggctcaag; r-tccgtcgttcgcttcatagg) Gabarap1L mice (f-catcgtggagaaggctccta; r-atacagctggcccatggtac); rat Map1lc3b (f-tttgtaagggcggttctgac; r-caggtagcaggaagcagagg). The housekeeping gene Rpl39 mice (f-caaaatcgccctattcctca; r-agacccagcttcgttctcct) and rat

Table 1
Effect of one CGRP injection (100 μg kg^{-1}) on metabolic and hormonal parameters in fed mice.

Parameters	Control	CGRP 15 min	CGRP 30 min	CGRP 60 min	p value
Glucose (mg/dl)	174 ± 9	271 ± 12 *	286 ± 15 *	260 ± 21 *	<0.05
Insulin (μUl/mL)	34.5 ± 8.9	7.1 ± 0.1 *	7.2 ± 0.3 *	8.0 ± 0.9 *	<0.05
Free fatty acids (mmol/L)	0.5 ± 0.06	0.5 ± 0.04	0.9 ± 0.07*	0.8 ± 0.06	<0.05
Cardiac Noradrenaline (ng/g)	1795 ± 104	1963 ± 204	1928 ± 216	2421 ± 299	>0.05
Plasma Adrenaline (ng/mL)	4.7 ± 0.7	7.2 ± 1.6	7.2 ± 2.8	5.3 ± 0.6	>0.05

Data are presented as mean ± SEM (n = 5).
ANOVA one-way, post-Tukey test.
* P < 0.05 vs Control treated with saline.

CyclophilinA (f-gcatacaggtcctggcatct; r-cttcccaaagagcacatgct) were used as a reference. The analysis was performed using ABI 7500 Prism software with the cycle threshold (Ct) value at which a statistically significant increase in signal occurred being used to ascertain expression level. Statistical analysis of results was performed using ΔΔ CT (Ct gene of interest – Ct housekeeping gene).

2.7. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). The statistical analysis of the results between the experimental groups was performed using the Student's "t" test, considering $p < 0.05$ as the level of significance. When necessary, ONE-WAY or TWO-WAY ANOVA test (post-Tukey test) was used, considering $p < 0.05$.

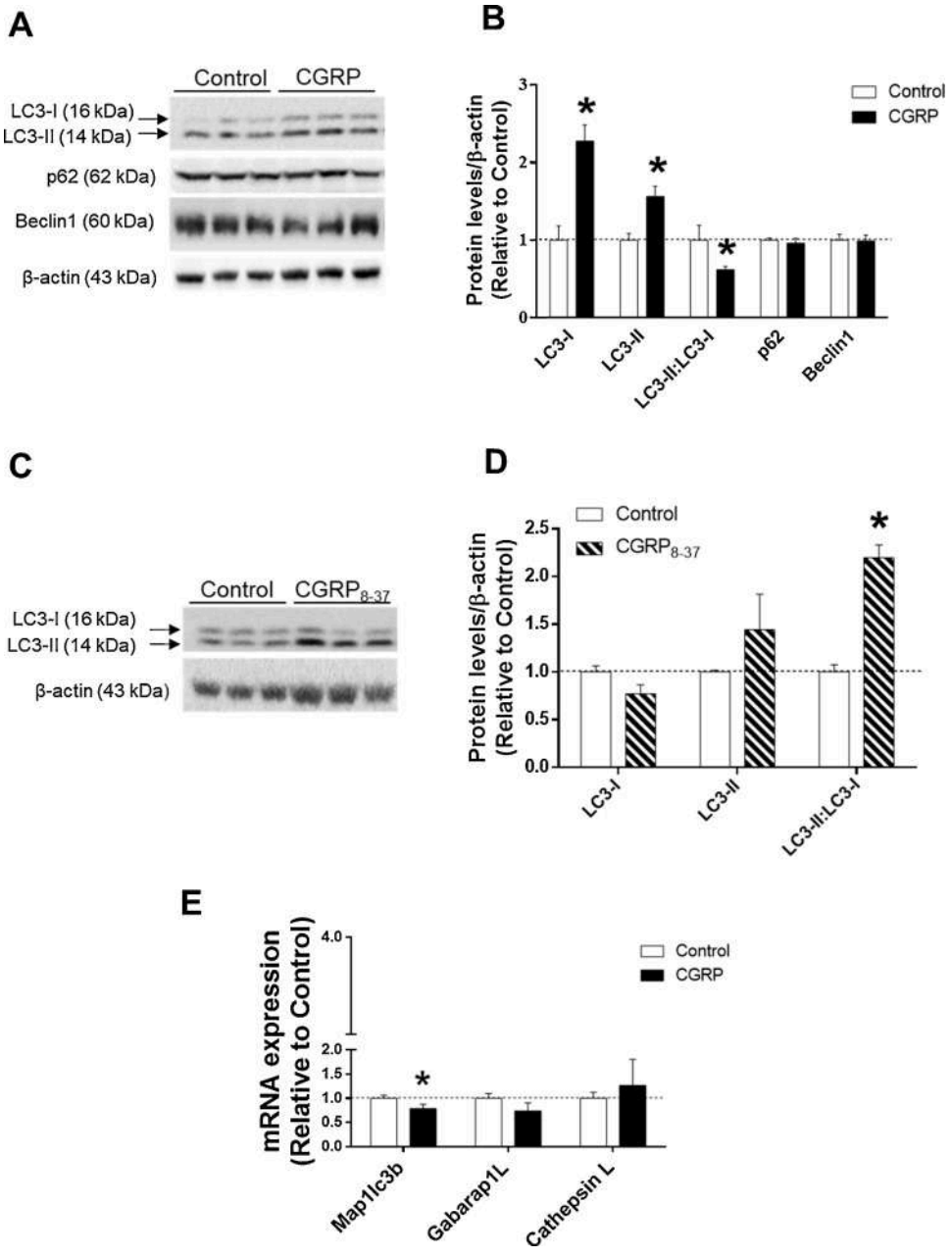

Fig. 1. CGRP inhibits autophagy in the heart of fed mice. (A, B) Protein content of the main autophagic markers: LC3-I, LC3-II, LC3-II:LC3-I, p62, and Beclin1 after 15 min of CGRP injection (100 μg kg^{-1}). (C, D) Protein content of LC3-I, LC3-II, and LC3-II:LC3-I after 45 min of the CGRP antagonist injection ($CGRP_{8-37}$, 100 μg kg^{-1}). (E) mRNA levels of autophagy-related genes: Map1lc3b, Gabarap1L, and Cathepsin L after 60 min of CGRP injection. Data are presented as the mean ± SE (n = 5); P < 0,05. * vs. Control.

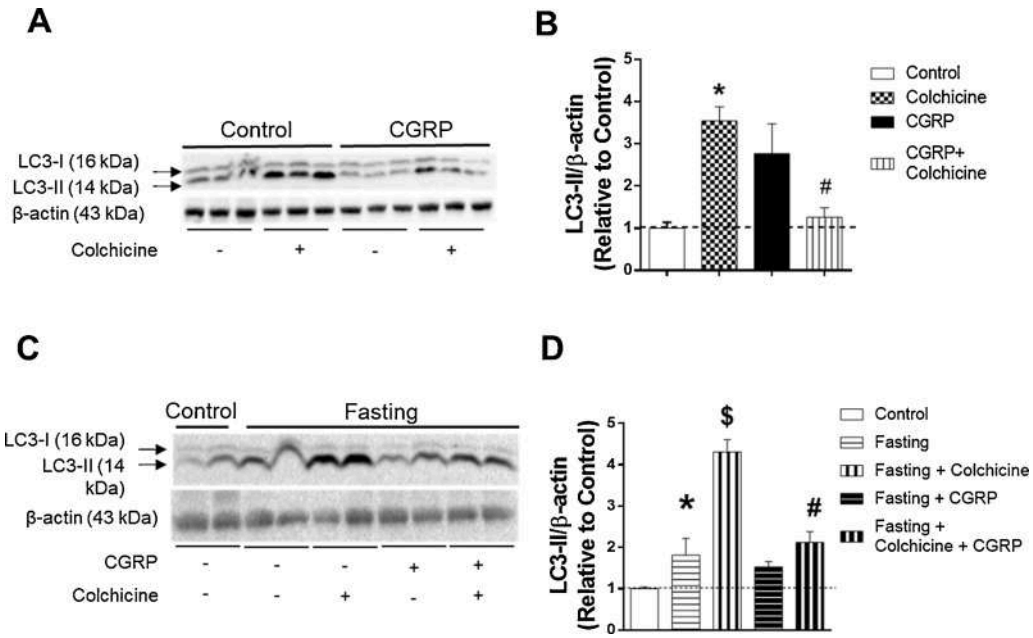

Fig. 2. CGRP suppresses basal and fasting-induced autophagy in mice hearts. Autophagic flux in the heart of (A, B) fed and (C, D) fasted mice for 24 h, after 15 min of CGRP injection (100 μg kg^{-1}). The animals received an injection of vehicle (saline solution) or 0.4 mg kg^{-1} colchicine administered at 48, 24, and 1 h before CGRP treatment. Data are presented as mean ± SE (n = 5); P < 0.05. * vs. Control; # vs. Colchicine/Fasting + Colchicine, $ vs. Fasting.

3. Results

3.1. Metabolic and hormonal parameters

As shown in Table 1, the acute treatment with CGRP at all-time intervals induced hyperglycemia. These effects are very likely due to the drastic drop (~80%) in the serum insulin levels caused by this peptide. Furthermore, CGRP for 30 min led to an increase in circulating free fatty acids indicating lipolysis. CGRP does not alter the content of cardiac norepinephrine and plasma epinephrine levels.

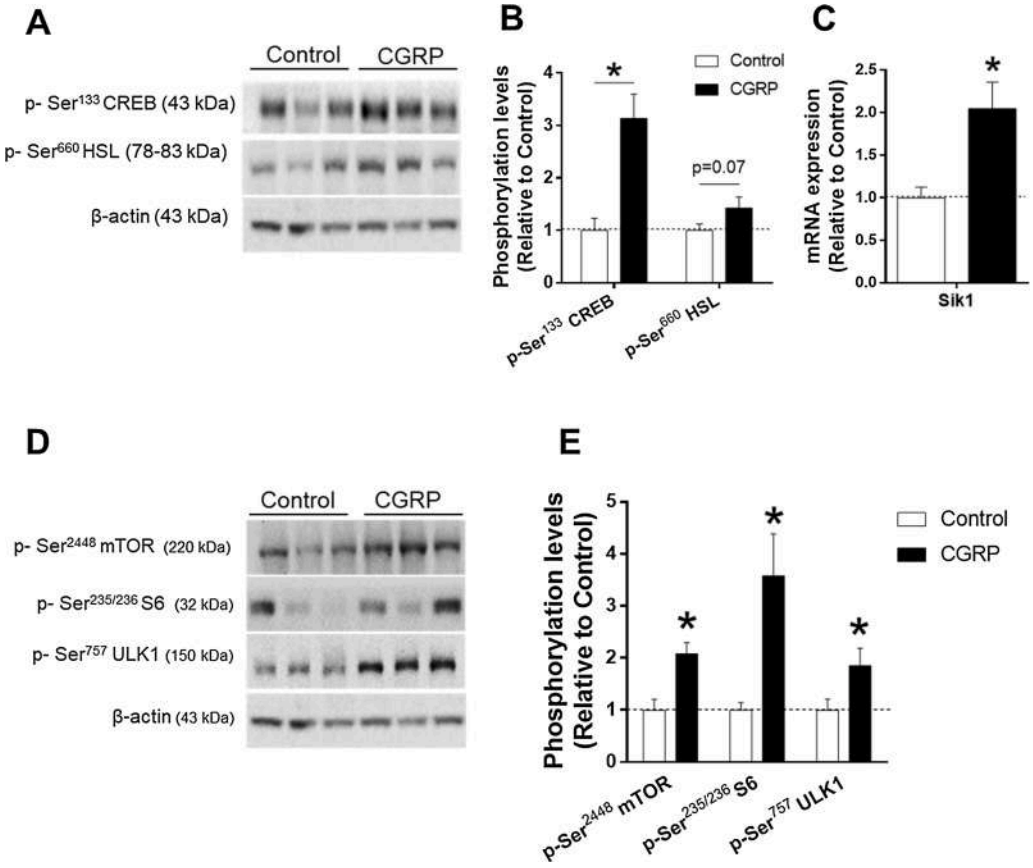

Fig. 3. CGRP activates PKA/CREB and mTOR signaling pathways in the heart of fed mice. (A, B) Phosphorylation state of PKA downstream targets (Ser^{133} CREB and Ser^{660} HSL). (C) Gene expression of Sik1. (D, E) Phosphorylation state of mTOR (Ser^{2448}) and their downstream targets ($Ser^{235/236}$ S6 and Ser^{757} ULK1). Proteins and genes were analyzed in the heart of fed mice after 15 and 60 min of CGRP injection (100 μg kg^{-1}), respectively. Data are presented as the mean ± SE; P < 0,05. * vs. Control.

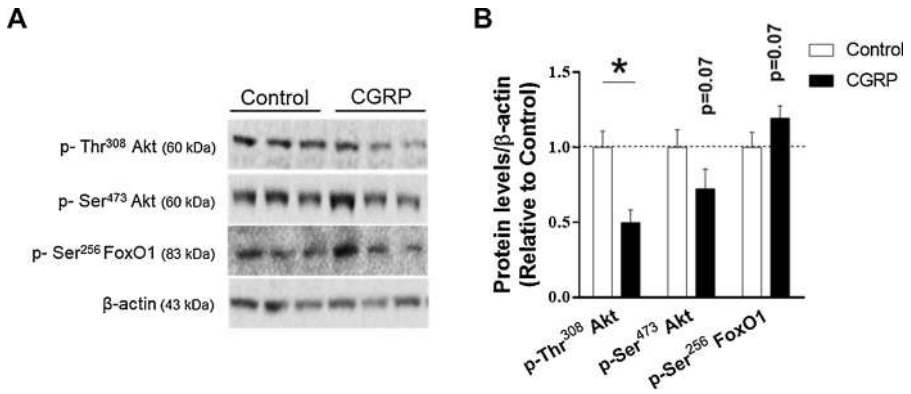

Fig. 4. CGRP suppresses Akt signaling pathway in the heart of fed mice (A, B) The phosphorylation state of Akt (Thr^{308} and Ser^{473}) and FoxO1 (Ser^{256}) in the heart of fed mice after 15 min of CGRP injection (100 μg kg^{-1}). Data are presented as the mean ± SE (n = 5); P < 0,05. * vs. Control.

3.2. CGRP inhibits autophagy in the mice heart under basal and fasted conditions

The acute effects of CGRP on the cardiac autophagic-lysosomal pathway were assessed by measurement of surrogate markers of this proteolytic process in fed mice 15 min after the treatment. It is known LC3-II is responsible for the formation and expansion of the autophagosome double membrane. Despite the increase in LC3-II content, CGRP treatment increased LC3-I (non-lipidated form) and decreased LC3-II: LC3-I ratio, a marker of committed autophagosome formation. Furthermore, we evaluated p62/SQSTM1 (sequestosome 1) levels, a protein present in autophagosome [18]. CGRP did not alter the protein content of either p62 or Beclin (Fig. 1A and B). To assess the endogenous effects of CGRP on cardiac autophagy, the CGRP pharmacological antagonist, CGRP$_{8\text{-}37}$, was administered. This treatment increased the protein content of LC3-II without altering the content of LC3-I. There was also an increase in the LC3-II:LC3-I ratio indicating a greater conversion from the non-lipidated to the lipidated form and autophagy activation (Fig. 1C and D). In addition, CGRP administration slightly decreased Map1lc3b expression (Fig. 1E) but did not affect other autophagy-related genes.

To confirm the inhibition of autophagy in the mouse heart, we treated fed animals with colchicine, a microtubule-disrupting agent that inhibits autophagosome-lysosome fusion. LC3-II accumulation after lysosomal inhibition reflects LC3-II flux or autophagy flux. Colchicine inhibited autophagosome degradation as identified by the increased protein content of LC3-II, and a single injection of CGRP completely abrogated this effect, 15 min before sacrifice (Fig. 2A and B).

To investigate CGRP in vivo effects on autophagic flux under a stimulated condition, animals were fasted for 24 h and were concomitantly treated with colchicine. As expected, fasting-induced a massive increase in autophagy flux, an effect that was abolished entirely by one CGRP single injection, 15 min before the sacrifice (Fig. 2C and D). Collectively, these data suggest that CGRP acutely inhibits autophagy in the heart of fed and fasted mice, probably through posttranscriptional mechanisms.

3.3. CGRP activates PKA/CREB and mTOR signaling pathways in mice cardiac muscle

Since most of the intracellular effects of CGRP in different tissues are mediated by PKA/CREB signaling, in vivo effects of CGRP on the phosphorylation levels of CREB and the HSL, two direct PKA targets, were investigated in the heart of fed animals. CGRP increased CREB Ser^{133} phosphorylation and tended to increase HSL Ser^{660} phosphorylation (p = 0.07) (Fig. 3A and B). Consistently, CGRP increased Sik1 mRNA levels (Fig. 3C), a well-known CREB target, indicating that CGRP directly activates PKA/CREB signaling in the cardiac muscle of fed animals. Because mTOR has recently been shown to be activated by PKA in other tissues [31] and plays a crucial role in inhibiting autophagy via ULK1, we hypothesized that this signaling pathway might be particularly important for the suppressive effects of CGRP on autophagy under basal conditions. CGRP increased mTOR Ser^{2448} phosphorylation, as well as its downstream targets, S6 Ser$^{235/236}$ and ULK1 Ser^{757} (Fig. 3D and E). Considering that Akt is a key protein involved in activating anabolic pathways via mTOR and inhibition of catabolic pathways related to autophagy via FoxO, the effect of CGRP treatment on the phosphorylation status of Akt and FoxO1 was evaluated. As shown in Fig. 4, CGRP treatment drastically decreased Akt phosphorylation in Ser^{473} residue but did not alter Thr^{308} residue (p = 0.07). FoxO1 Ser^{256} phosphorylation in the heart of animals treated with CGRP was also not affected (Fig. 4A and B). Taken together, these results strongly suggest that CGRP in vivo inhibits autophagy via PKA and mTOR signaling in cardiac muscle of fed animals.

3.4. CGRP activates cAMP, mTOR, and Akt signaling pathways in cardiomyocytes

To investigate further the signaling pathways involved in CGRP suppressive effect on autophagy, cAMP, mTOR, and Akt signaling were assessed in cardiomyocytes under fed conditions. In fed cardiomyocytes, the addition of CGRP (1μM) to the incubation medium, during 15 min, increased PKA substrates (~2-fold) and CREB Ser^{133} (~7-fold) phosphorylation levels, indicating increased cAMP cascade activity (Fig. 5A and B). CGRP also activated the mTOR pathway as estimated by the high levels of S6 (Fig. 5E and F). In contrast to in vivo findings, CGRP in vitro drastically increased Akt phosphorylation levels at both residues (Thr^{308} and Ser^{473}), and FoxO1 Ser^{256} and FoxO4 Ser^{193} (Fig. 5C and D). In addition, the incubation with CGRP for 60 min led to Map1lc3b expression decrease (50 %) (Fig. 5G). To further investigate the inhibitory effect of CGRP on autophagy, cardiomyocytes were incubated with chloroquine, a potent inhibitor of autophagic flux, for 4 h and treated with CGRP for 15 min. As shown in Fig. 6, CGRP decreases the protein content of LC3-II in the presence of chloroquine, strongly indicating the inhibitory action of CGRP on cardiac autophagy.

4. Discussion

In previous studies, we have shown the Sympathetic Nervous System, through the activation of the Gs-coupled β-AR and the cAMP/PKA signaling cascade, suppresses proteolysis and autophagy in skeletal muscles [32–36] and brown adipose tissue of rodents [37]. Like catecholamines, CGRP is a neuropeptide that activates PKA/CREB signaling pathway, as here demonstrated in mice hearts and cardiomyocytes. Although several CGRP effects on the heart have already been reported [8,9,38–42], to our knowledge, the present study is the first to

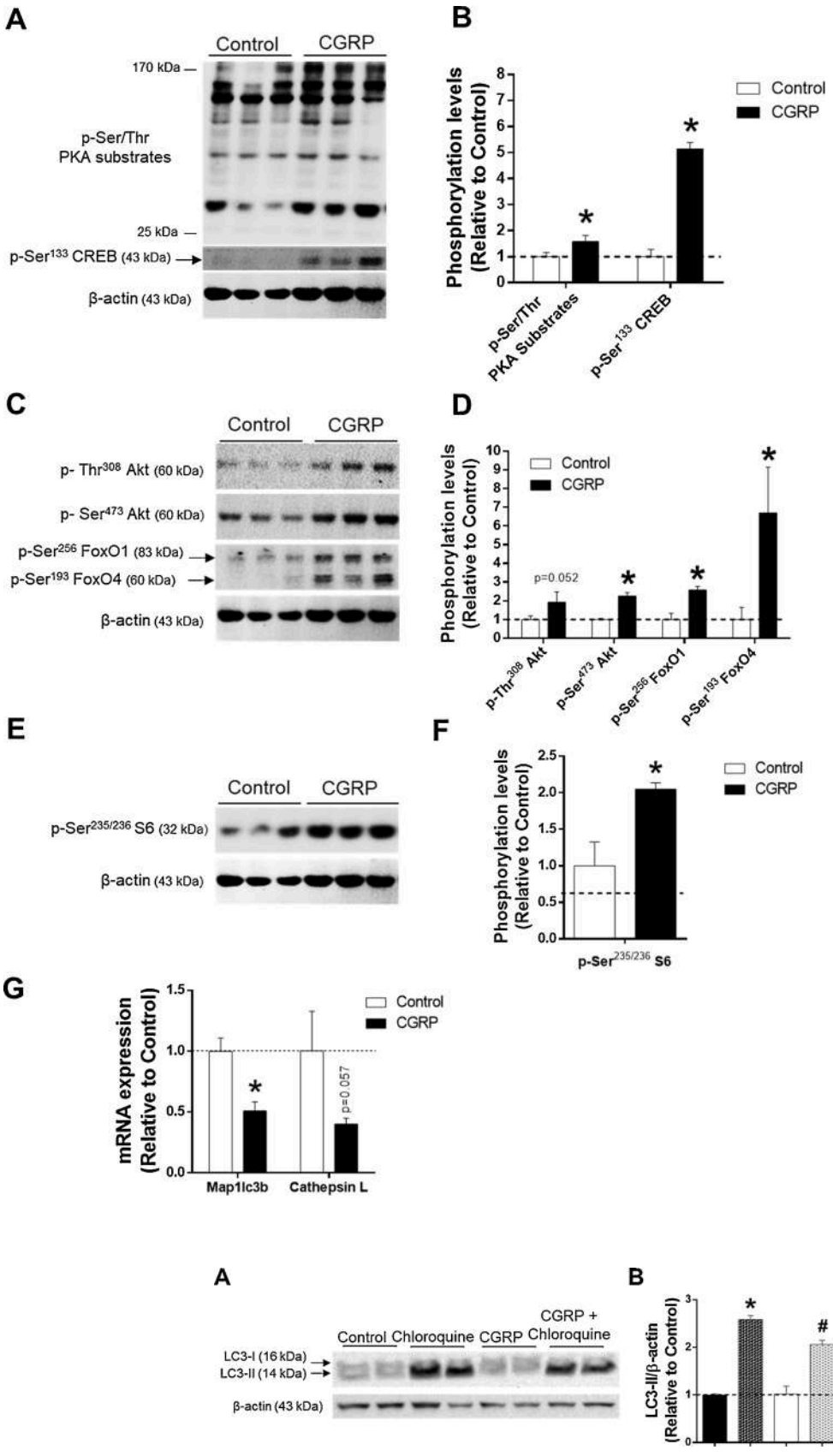

Fig. 5. CGRP directly activates PKA/CREB, AKT/FoxO1, and mTOR signaling pathways in cardiomyocytes. Phosphorylation levels of (A, B) PKA substrates and CREB (Ser^{133}); (C, D) Akt (Thr^{308} and Ser^{473}) and FoxO (Ser^{256} and Ser^{193}); and (E, F) S6 ($Ser^{235/236}$) in cardiomyocytes incubated with or not CGRP (1μM) during 15 min. (G) mRNA levels of autophagic-related genes: Map1lc3b and CathepsinL in cardiomyocytes incubated with CGRP (1μM) during 60 min. Data are presented as the mean ± SE (n = 3); P < 0,05. * vs. Control.

Fig. 6. CGRP inhibitis autophagic flux in cardiomyocytes. Cells were for 4 h incubated with chloroquine (40 μM) and CGRP (1μM) for 15 min. Data are presented as the mean ± SE (n = 3); P < 0,05. * vs Control; # vs. Chloroquine.

demonstrate that this neuropeptide plays a modulatory role in mice cardiac autophagy. The data show that CGRP acutely inhibited autophagy in hearts from fed mice as estimated by decrease in the LC3-II: LC3-I ratio, autophagic flux and expression of Map1lc3b. Similar effects were observed in cardiomyocytes incubated with the peptide. Conversely, LC3-II:LC3-I ratio was increased in fed animals treated with $CGRP_{8-37}$, a selective CGRP antagonist, suggesting an endogenous inhibitory effect of CGRP on cardiac autophagy. In the setting of a catabolic situation, our data demonstrate that one single injection of CGRP completely abolished the fasting-driven autophagic flux activation. These results indicate that CGRP suppresses cardiac autophagy by transcriptional (autophagic genes) and/or post-translational (LC3 lipidation) mechanisms and are fully in line with previous studies carried out by Machado et al. [13,14], who demonstrated similar effects of CGRP on skeletal muscle of rodents, both in basal and atrophic conditions. More recently, Tian et al. (2020) has also demonstrated that treatment with CGRP for seven days decreased neural autophagy after brain injury, corroborating the results presented here [43].

It is well-established that Akt is a critical protein in the regulation of protein metabolism because, in addition to stimulating protein synthesis and inhibiting autophagy via mTOR, it promotes phosphorylation and inactivation of FoxO, a transcriptional factor that activates atrophic and autophagy-related genes transcription [44,45]. Our in vivo data demonstrate that CGRP drastically reduced Akt phosphorylation status. The opposite was observed in vitro, where CGRP drastically increased the phosphorylation of this kinase and FoxO. In this context, it is possible to speculate Akt inhibition observed in response to in vivo treatment is an indirect effect due to low plasma levels of insulin. That CGRP inhibits insulin secretion and promotes hyperglycemia has already been demonstrated in other studies. Pettersson et al. (1986) were the first to show that, after 6 min of intravenous administration of CGRP (3.2 μg/kg) in rats, there is an increase in glycemia associated with a significant decrease in plasma insulin [46]. Ishizuka et al. (1988) observed a direct inhibitory action of CGRP on insulin release in pancreatic cell culture, but the underlying mechanisms are still not known [47]. Unlike Akt, our data also show that CGRP in vivo did not alter the phosphorylation status of FoxO1. A reasonable explanation for these controversial data is that CGRP via PKA could phosphorylate FoxO and thus counteract the inhibition of Akt promoted by hypoinsulinemia. The experiments carried out by Silveira et al. (2014) in skeletal muscles and by Lee et al. (2011) in endothelial cells demonstrated that PKA directly phosphorylates FoxO1 independently of Akt [36,48]. Together, these data indicate that the Akt/FoxO signaling pathway does not mediate the inhibitory in vivo effects of CGRP on cardiac autophagy in fed mice and probably involves a posttranslational mechanism.

mTOR is a Ser/Thr kinase involved in the regulation of protein synthesis, cell growth, ribosomal biogenesis, etc. Also, it is well established that mTOR inhibits autophagy, an effect mediated by inhibitory phosphorylation in $ULK1^{Ser757}$, a critical protein in the initiation of autophagosome formation [19]. Our findings show an increase in mTOR, S6, and $ULK1^{Ser757}$ phosphorylation in the heart of mice, just after 15 min of treatment with CGRP. In total agreement with these data, CGRP drastically increased p-S6 in fed cardiomyocytes. The activation of mTOR signaling in response to CGRP in vivo is independent of the action of Akt and probably mediated by PKA. In fact, Liu et al. (2016) has convincingly shown that mTOR is triggered by PKA activation in both mouse and human brown adipocytes, an independent effect of insulin/Akt signaling [49]. Similar effects have been reported in skeletal muscles and other tissues. For example, the Thyroid Stimulating Hormone (TSH) has been shown to activate p70 ribosomal S6 kinase (S6K1) through mTOR in the thyroid epithelium in a cAMP-dependent manner [31]. Considering mTOR as the main inhibitor of autophagic-lysosomal proteolysis [50,51], and knowing that CGRP directly inhibits this proteolytic system in rat skeletal muscles and C2C12 cells [13,14], the present data suggest the CGRP capacity to reduce autophagy in the heart is due to the activation of mTOR via PKA, leading to phosphorylation and inhibition of $ULK1^{Ser757}$.

These CGRP rapid effects can be explained by the short half-life of the peptide (~10 min) [26] and by the interaction between GPCR-like receptor and b-arrestin, a protein that causes internalization of these receptors, after their coupling to its agonist, and leading the rapid interruption of signal transduction [52].

5. Conclusion

In summary, our findings demonstrate the acute effects of CGRP in the inhibition of cardiac autophagy. Probably, these effects are mediated by mTOR signaling independently of Akt. Further studies are needed to evaluate the potential benefits or not of inhibiting cardiac autophagy by CGRP in pathological conditions.

CRediT authorship contribution statement

Aline Zanatta Schavinski: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Juliano Machado:** Methodology, Investigation, Writing - original draft, Visualization. **Henrique Jorge Novaes Morgan:** Methodology, Investigation. **Natalia Lautherbach:** Investigation. **Silvia Paula-Gomes:** Investigation. **Isis C. Kettelhut:** Writing - review & editing, Funding acquisition. **Luiz Carlos C. Navegantes:** Supervision, Conceptualization, Methodology, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

[1] S.G. Amara, V. Jonas, M.G. Rosenfeld, E.S. Ong, R.M. Evans, Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products, Nature 298 (5871) (1982) 240–244, https://doi.org/10.1038/298240a0. Available from:.

[2] F.A. Russell, R. King, S.-J. Smillie, X. Kodji, S.D. Brain, Calcitonin gene-related peptide: physiology and pathophysiology, Physiol. Rev. 94 (4) (2014) 1099–1142. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25287861.

[3] C.A. Maggi, Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves, Prog. Neurobiol. 45 (1) (1995) 1–98. Available from: https://www.sciencedirect.com/science/article/pii/0301008294E0017B?via%3Dihub.

[4] I.L. Gibbins, J.B. Furness, M. Costa, I. MacIntyre, C.J. Hillyard, S. Girgis, Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs, Neurosci. Lett. 57 (2) (1985) 125–130. Available from: https://www.sciencedirect.com/science/article/abs/pii/0304394085900503.

[5] N. Aiyar, K. Rand, N.A. Elshourbagy, Z. Zeng, J.E. Adamou, D.J. Bergsma, et al., A cDNA encoding the calcitonin gene-related peptide type 1 receptor, J. Biol. Chem. 271 (19) (1996) 11325–11329. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8626685.

[6] R. Iyengar, J.D. Hildebrandt, G Protein Pathways. Volume 344, Part B, G Proteins and their Regulators, Elsevier, 2001, p. 719.

[7] C. Gennari, J.A. Fischer, Cardiovascular action of calcitonin gene-related peptide in humans, Calcif. Tissue Int. 37 (6) (1985) 581–584. Available from: https://pubmed.ncbi.nlm.nih.gov/3937576/.

[8] X. Wang, R.R. Fiscus, Calcitonin gene-related peptide increases cAMP, tension, and rate in rat atria, Am. J. Physiol. 256 (2 Pt 2) (1989) R421–8.

[9] S.D. Brain, A.D. Grant, Vascular actions of calcitonin gene-related peptide and adrenomedullin, Physiol. Rev. 84 (3) (2004) 903–934, https://doi.org/10.1152/physrev.00037.2003. Available from:.

[10] Y.J. Li, Z.S. Xiao, C.F. Peng, H.W. Deng, Calcitonin gene-related peptide-induced preconditioning protects against ischemia-reperfusion injury in isolated rat hearts, Eur. J. Pharmacol. 311 (2–3) (1996) 163–167.

[11] D. Bell, M. Tamamori, F. Marumo, M. Hiroe, B.J. McDermott, H. Ito, Calcitonin gene-related peptide (CGRP) increases cell surface area and induces expression of skeletal alpha-actin ANP mRNA in hypertrophying neonatal cardiomyocytes, Regul. Pept. 71 (1) (1997) 1–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9299635.

[12] H. Ito, D. Bell, M. Tamamori, T. Nozato, T. Shimojo, S. Adachi, et al., Calcitonin gene-related peptide (CGRP) and hypertrophy of cardiomyocytes, Heart Vessels (Suppl. 12) (1997) 15–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9476534.

[13] J. Machado, L.H. Manfredi, W.A. Silveira, D.A.P. Gonçalves, D. Lustrino, N. M. Zanon, et al., Calcitonin gene-related peptide inhibits autophagic-lysosomal proteolysis through cAMP/PKA signaling in rat skeletal muscles, Int. J. Biochem. Cell Biol. 72 (2016) 40–50.

[14] J. Machado, W.A. Silveira, D.A. Gonçalves, A.Z. Schavinski, M.M. Khan, N. M. Zanon, et al., A−calcitonin gene-related peptide inhibits autophagy and calpain systems and maintains the stability of neuromuscular junction in denervated muscles, Mol. Metab. (2019). Available from: https://www.sciencedirect.com/science/article/pii/S2212877819302546.

[15] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (7182) (2008) 1069–1075. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18305538.

[16] B. Ravikumar, S. Sarkar, J.E. Davies, M. Futter, M. Garcia-Arencibia, Z.W. Green-Thompson, et al., Regulation of mammalian autophagy in physiology and pathophysiology, Physiol. Rev. 90 (4) (2010) 1383–1435, https://doi.org/10.1152/physrev.00030.2009. Available from:.

[17] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (1) (2008) 27–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18191218.

[18] D.J. Klionsky, D.J. Klionsky, K. Abdelmohsen, A. Abe, M.J. Abedin, H. Abeliovich, et al., Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), Autophagy 12 (1) (2016) 1–222.

[19] D. Egan, J. Kim, R.J. Shaw, K.-L. Guan, The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR, Autophagy 7 (6) (2011) 643–644. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21460621.

[20] J. Kim, M. Kundu, B. Viollet, K.-L. Guan, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell Biol. 13 (2) (2011) 132–141. Available from: http://www.nature.com/articles/ncb2152.

[21] Y. Zhao, J. Yang, W. Liao, X. Liu, H. Zhang, S. Wang, et al., Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity, Nat. Cell Biol. 12 (7) (2010) 665–675.

[22] C. Mammucari, G. Milan, V. Romanello, E. Masiero, R. Rudolf, P. Del Piccolo, et al., FoxO3 controls autophagy in skeletal muscle in vivo, Cell Metab. 6 (6) (2007) 458–471. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18054315.

[23] D.R. Alessi, P. Cohen, Mechanism of activation and function of protein kinase B, Curr. Opin. Genet. Dev. 8 (1) (1998) 55–62. Available from: https://www.sciencedirect.com/science/article/pii/S0959437X98800622?via%3Dihub.

[24] D.A. Gonçalves, W.A. Silveira, L.H. Manfredi, F.A. Graça, A. Armani, E. Bertaggia, et al., Insulin/IGF1 signalling mediates the effects of β 2 -adrenergic agonist on muscle proteostasis and growth, J. Cachexia Sarcopenia Muscle 10 (2) (2019) 455–475. Available from: https://pubmed.ncbi.nlm.nih.gov/30932373/.

[25] W.A. Silveira, D.A. Gonçalves, J. Machado, N. Lautherbach, D. Lustrino, S. Paula-Gomes, et al., cAMP-dependent protein kinase inhibits FoxO activity and regulates skeletal muscle plasticity in mice, FASEB J. 34 (9) (2020) 12946–12962, 1. Available from: https://pubmed.ncbi.nlm.nih.gov/32772437/.

[26] M.E. Kraenzlin, J.L. Ch'ng, P.K. Mulderry, M.A. Ghatei, S.R. Bloom, Infusion of a novel peptide, calcitonin gene-related peptide (CGRP) in man. Pharmacokinetics and effects on gastric acid secretion and on gastrointestinal hormones, Regul. Pept. 10 (2–3) (1985) 189–197. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3922013.

[27] J.-S. Ju, A.S. Varadhachary, S.E. Miller, C.C. Weihl, Quantitation of " autophagic flux" in mature skeletal muscle, Autophagy 6 (7) (2010) 929–935. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20657169.

[28] N.N. Kim, F.J. Villarreal, M.P. Printz, A.A. Lee, W.H. Dillmann, Trophic effects of angiotensin II on neonatal rat cardiac myocytes are mediated by cardiac fibroblasts, Am. J. Physiol. – Endocrinol. Metab. 269 (1995) (3 32-3). Available from: https://pubmed.ncbi.nlm.nih.gov/7573419/.

[29] M.A. Garofalo, I.C. Kettelhut, J.E. Roselino, R.H. Migliorini, Effect of acute cold exposure on norepinephrine turnover rates in rat white adipose tissue, J. Auton. Nerv. Syst. 60 (3) (1996) 206–208. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8912272.

[30] S. Paula-Gomes, D. Gonçalves, A. Baviera, N. Zanon, L. Navegantes, I. Kettelhut, Insulin suppresses atrophy- and autophagy-related genes in heart tissue and cardiomyocytes through AKT/FOXO signaling, Horm. Metab. Res. 45 (12) (2013) 849–855. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23757118.

[31] S. Blancquaert, L. Wang, S. Paternot, K. Coulonval, J.E. Dumont, T.E. Harris, et al., cAMP-dependent activation of mammalian target of rapamycin (mTOR) in thyroid cells. Implication in mitogenesis and activation of CDK4, Mol. Endocrinol. 24 (7) (2010) 1453–1468. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20484410.

[32] A.M. Baviera, N.M. Zanon, L.C. Carvalho Navegantes, R.H. Migliorini, Kettelhut I do C. Pentoxifylline inhibits Ca 2+ -dependent and ATP proteasome-dependent proteolysis in skeletal muscle from acutely diabetic rats, Am. J. Physiol. Metab. 292 (3) (2007) E702–708. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17077345.

[33] D.A.P. Gonçalves, W.A. Silveira, E.C. Lira, F.A. Graça, S. Paula-Gomes, N.M. Zanon, et al., Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt, Am. J. Physiol. Metab. 302 (1) (2012) E123–133, https://doi.org/10.1152/ajpendo.00188.2011. Available from:.

[34] F.A. Graça, D.A.P. Gonçalves, W.A. Silveira, E.C. Lira, V.E. Chaves, N.M. Zanon, et al., Epinephrine depletion exacerbates the fasting-induced protein breakdown in fast-twitch skeletal muscles, Am. J. Physiol. Metab. 305 (12) (2013) E1483–1494. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24169047.

[35] L.C.C. Navegantes, R.H. Migliorini, I. do Carmo Kettelhut, Adrenergic control of protein metabolism in skeletal muscle, Curr. Opin. Clin. Nutr. Metab. Care 5 (3) (2002) 281–286. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11953653.

[36] W.A. Silveira, D.A. Gonçalves, F.A. Graça, A.L. Andrade-Lopes, L.B. Bergantin, N. M. Zanon, et al., Activating cAMP/PKA signaling in skeletal muscle suppresses the ubiquitin-proteasome-dependent proteolysis: implications for sympathetic regulation, J. Appl. Physiol. 117 (1) (2014) 11–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24833777.

[37] F. Przygodda, N. Lautherbach, S.L. Buzelle, D.A. Goncalves, A.P. Assis, S. Paula-Gomes, et al., Sympathetic innervation suppresses the autophagic-lysosomal system in brown adipose tissue under basal and cold-stimulated conditions, J. Appl. Physiol. 128 (4) (2020) 855–871.

[38] S.D. Brain, T.J. Williams, J.R. Tippins, H.R. Morris, I. MacIntyre, Calcitonin gene-related peptide is a potent vasodilator, Nature 313 (5997) (1985) 54–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3917554.

[39] H. Ito, D. Bell, M. Tamamori, T. Nozato, T. Shimojo, S. Adachi, et al., Calcitonin gene-related peptide (CGRP) and hypertrophy of cardiomyocytes, Heart Vessels (Suppl. 12) (1997) 15–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9476534.

[40] M. Al-Rubaiee, P.R. Gangula, R.M. Millis, R.K. Walker, N.A. Umoh, V.M. Cousins, et al., Inotropic and lusitropic effects of calcitonin gene-related peptide in the heart, Am. J. Physiol. Circ. Physiol. 304 (11) (2013) H1525–1537, https://doi.org/10.1152/ajpheart.00874.2012. Available from:.

[41] D. Bell, K.D. Schlüter, X.J. Zhou, B.J. McDermott, H.M. Piper, Hypertrophic effects of calcitonin gene-related peptide (CGRP) and amylin on adult mammalian ventricular cardiomyocytes, J. Mol. Cell. Cardiol. 27 (11) (1995) 2433–2443. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8596194.

[42] J. Li, S.P. Levick, D.J. DiPette, J.S. Janicki, S.C. Supowit, Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure, Regul. Pept. 185 (2013) 20–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23816470.

[43] J. Tian, L. Yang, P. Wang, L. Yang, Z. Fan, Exogenous CGRP regulates apoptosis and autophagy to alleviate traumatic brain injury through Akt/mTOR signalling pathway, Neurochem. Res. 45 (12) (2020) 2926–2938, https://doi.org/10.1007/s11064-020-03141-9. Available from:.

[44] J.-W. Lee, H. Chen, P. Pullikotil, M.J. Quon, Protein kinase A-α directly phosphorylates FoxO1 in vascular endothelial cells to regulate expression of vascular cellular adhesion molecule-1 mRNA, J. Biol. Chem. 286 (8) (2011) 6423–6432, https://doi.org/10.1074/jbc.M110.180661. Available from:.

[45] G. OUDIT, H. Sun, B.-G. Kerfant, M.A. Crackower, J.M. Penninger, P.H. Backx, The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease, J. Mol. Cell. Cardiol. 37 (2) (2004) 449–471. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15276015.

[46] M. Pettersson, B. Ahrén, G. Böttcher, F. Sundler, Calcitonin gene-related peptide: occurrence in pancreatic islets in the mouse and the rat and inhibition of insulin secretion in the mouse, Endocrinology 119 (2) (1986) 865–869.

[47] J. Ishizuka, G.H. Greeley, C.W. Cooper, J.C. Thompson, Effect of calcitonin gene-related peptide on glucose and gastric inhibitory polypeptide-stimulated insulin release from cultured newborn and adult rat islet cells, Regul. Pept. 20 (1) (1988) 73–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3281189.

[48] J.-W. Lee, H. Chen, P. Pullikotil, M.J. Quon, Protein kinase A-α directly phosphorylates FoxO1 in vascular endothelial cells to regulate expression of vascular cellular adhesion molecule-1 mRNA, J. Biol. Chem. 286 (8) (2011) 6423–6432, https://doi.org/10.1074/jbc.M110.180661. Available from:.

[49] D. Liu, M. Bordicchia, C. Zhang, H. Fang, W. Wei, J.-L. Li, et al., Activation of mTORC1 is essential for β-adrenergic stimulation of adipose browning, J. Clin. Invest. 126 (5) (2016) 1704–1716. Available from: https://www.jci.org/articles/view/83532.

[50] C. Settembre, R. Zoncu, D.L. Medina, F. Vetrini, S. Erdin, S. Erdin, et al., A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB, EMBO J. 31 (5) (2012) 1095–1108. Available from: https://pubmed.ncbi.nlm.nih.gov/22343943/.

[51] J. Zhou, S.H. Tan, V. Nicolas, C. Bauvy, Yang N. Di, J. Zhang, et al., Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion, Cell Res. 23 (4) (2013) 508–523. Available from: https://pubmed.ncbi.nlm.nih.gov/23337583/.

[52] J.J. Gingell, E.R. Hendrikse, D.L. Hay, New insights into the regulation of CGRP-family receptors, Trends Pharmacol. Sci. 40 (1) (2019) 71–83. Available from: https://www.sciencedirect.com/science/article/pii/S0165614718302116#bib0015.