Current Topics in Pharmacology
Vol. 26, 2022

Original Article

# Role of autonomic nervous system in the inotropic effect of obestatin

**Bilyana Ilieva, Mariela Chichova, Hristo Gagov and Iliyana Sazdova***

Department of Human and Animal Physiology, Faculty of Biology, Sofia University 'St. Kliment Ohridski', Sofia, Bulgaria.

## ABSTRACT

Obestatin is a gastrointestinal hormone with various peripheral and central effects. It induces a positive inotropic effect on excised frog heart preparation, which consists of cardiac muscle tissue with preserved endocardium and functionally active projections of autonomic neurons. The hypothesis that obestatin targets sympathetic nerve endings was checked. The inhibitor of vesicular monoamine transporter reserpine (10 μM), norepinephrine reuptake inhibitor desipramine (1 μM) and tyrosine hydroxylase inhibitor 3-Iodothyrosine (3-IT; 0.02 mg/g b.w.) were administered prior to obestatin application to study the involvement of each of these molecules in the observed physiological response. The proper adrenergic signaling was tested at the end of experiments with 50 μM epinephrine. The positive inotropic effect of obestatin was abolished in the presence of 3-IT and reserpine, while desipramine just slightly reduced the observed effect (by up to 50%). It is concluded that the positive inotropic effect of obestatin is mediated by a neuronal-dependent mechanism. It is suggested that this regulation is probably realized mainly by an increase in epinephrine secretion.

**KEYWORDS:** obestatin, heart, adrenergic, sympathetic.

*Corresponding author: i.sazdova@biofac.uni-sofia.bg

## INTRODUCTION

Obestatin is a 23-amino acid ghrelin-associated peptide derived from the posttranslational processing of preproghrelin [1]. Preproghrelin is a polypeptide containing 117 amino acid residues encoded by the ghrelin gene [2]. The end products of this gene are acylated ghrelin (acyl ghrelin), unacylated ghrelin (des-acyl ghrelin) and obestatin. The latter regulates mainly energy intake and metabolism [3].

Obestatin has a positive effect on cardiovascular function [4]. It has been shown to modulate the heart force of contraction, vascular tone as well as to reduce cell death and cardiomyocyte apoptosis after ischemia/reperfusion [5]. These effects are mediated by a still unknown G-protein coupled receptors, [6] which activate phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and extracellular signal regulated kinases 1/2 (ERK 1/2) downstream [7-9]. It has also been found that the beneficial effect of obestatin against reduced contractility and beta-adrenergic response may relate to its ability to up-regulate beta-adrenergic receptors and α-myosin heavy chain in the heart of diabetic rats [5, 10].

Also, it was shown that the cardiovascular effects of obestatin on papillary muscles and on the whole heart are mediated by obestatin-induced activation of PI3K/Akt/NO/cyclic GMP-dependent protein kinase (PKG) signaling pathway and that this mechanism is involved in the protection of the myocardium, caused by adrenergic, endothelinergic or ischemia/reperfusion stress [11].

However, the mechanisms by which obestatin regulates cardiac contractility remain largely unknown.

We have previously shown an obestatin-dependent increase in maximal force of contraction of excised frog hearts [8]. It was suggested that the positive inotropic effect of obestatin, induced by PI3K-, PKC- and ERK1/2-dependent, and pertussis toxin-sensitive mechanism, was due to increase in catecholamine secretion or because of sensitized adrenergic signaling in cardiomyocytes. In amphibians, epinephrine is the main sympathetic neurotransmitter, whereas norepinephrine dominates in the circulation [12]. In order to clarify which of these alternatives is responsible for the observed physiological effect we tested the hypothesis on indirect obestatin regulation of frog heart function *via* sympathetic nerve endings. For this purpose, we inhibited three steps of epinephrine synthesis, namely a rate-limiting reaction in this synthesis, loading of secretory vesicles and epinephrine reuptake by using appropriate pharmacological tools.

## MATERIALS AND METHODS

### Experimental animals

All experimental procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Bulgarian Center for Bioethics. *Pelophylax ridibundus* frogs with a body weight of approximately 40-50 g were used. The animals were denervated and the hearts were cannulated and isolated by the Straub method in all experimental groups.

### Experimental procedures

All experiments were performed at room temperature (20-22 $^{o}$C). Frogs were placed in a bell jar with anesthetic-soaked cotton (ethyl ether). Frogs were killed by double pithing and their hearts were excised and cannulated. The cannula was passed via the left aortic branch (*truncus arteriosus sinister*) and aortic trunk (*conus arteriosus*), and then was inserted into the ventricle. The volume of the cannula is approximately 500 µl. The hearts were connected to highly sensitive force-displacement transducer GRASS FT03 (Grass Instrument Co., Quincy, USA). Contractions were recorded and analyzed on a computer using interface and TENZOSU software (Stocks, Sofia, Bulgaria).

Reserpine (10 µM) and desipramine (1 µM) were diluted in Ringer solution and were administered at a final volume of 200 µl into the cannula at least 25 minutes prior to obestatin administration. The inhibitors were also present at all subsequent administrations and along with obestatin until the end of the experiment. 3-Iodothyrosine (3-IT) was applied *in vivo* as an injection into the dorsal lymph sac at a dose of 0.02 mg/g b.w. one hour prior to the isolation of hearts. The proper signaling of adrenergic receptors on the heart muscle cell membranes was tested by the administration of 50 µM epinephrine during the last 15 minutes of each experiment.

### Drugs and reagents

All substances were dissolved in modified Ringer solution with the following composition – 100 mmol/l NaCl, 1.3 mmol/l KCl, 0.7 mmol/l $CaCl_2$, 5 mmol HEPES and 1.2 mmol/l $NaHCO_3$, pH = 7. This solution (200 µl) was introduced into the frog heart ventricle using a cannula. The injections of pure or inhibitor-containing Ringer solution were given at fixed intervals during 75 min experiments. The tyrosine hydroxylase (TH) inhibitor 3-IT was injected 1 hour prior to the experiment to provide sufficient time for its effect.

Obestatin was obtained from Bachem AG, Switzerland and 3-iodothyrosine, reserpine, desipramine, epinephrine and all salts were from Sigma-Aldrich Inc., USA.

### Statistical analysis

Data obtained from each experimental animals are expressed as percent of the initial contractile force, measured 4 min from the beginning of the experiment, which was taken as 100%. All data are presented as means ± SEM. The *n* refers to the number of frog heart preparations. Statistical significance was determined by Student's t test for independent samples; $p<0.05$ was considered statistically significant.

## RESULTS

The maximal force of contractions moderately decreases in the first 15-20 min of the experiment.

Thereafter the spontaneous contractions of excised frog hearts remain stable for a long time [8]. We applied two concentrations of obestatin (1 and 100 nmol/l) at 15 min intervals. Higher concentration of 100 nmol/l guaranteed maximal response needed for the proper cell signaling study [8].

The positive inotropic effect of obestatin was abolished in the presence of 3-IT, a selective inhibitor of TH that catalyzes the rate-limiting reaction in the catecholamine biosynthesis pathway [13] (Figure 1). The amplitude of maximal force of heart contractions in the presence of obestatin after pre-treatment with 3-IT is similar to that in control conditions ($p \geq 0.114$, not shown).

The effect of obestatin on the maximal force of contraction was completely inhibited in the presence of reserpine, an irreversible blocker of vesicular monoamine transporter (VMAT) [14], (Figure 2).

The role of catecholamine reuptake in the positive inotropic effect of obestatin was tested by the application of selective epinephrine transporter inhibitor desipramine [15-17] (Figure 3). An approximately 50% reduction of positive inotropic effect of obestatin after simultaneous application of desipramine and obestatin was observed (Table 1).

## DISCUSSION

Excised frog heart consists of cardiac muscle tissue with its endocardium and functionally active projections of autonomic neurons. These *in vitro* preparations develop regular contractions with stable pattern and force [8]. Presynaptic denervation of the frog's sympathetic ganglion leads to impaired synaptic transmission within 24-48 hours. Postganglion cells did not show noticeable structural changes, but an increase in membrane resistance was observed later in the course of denervation [18]. Isolated frog hearts have long been known as a very convenient experimental model for various physiological and pharmacological studies [9, 19]. The axons of autonomic neurons in the wall of amphibian heart are composed of long chains with varicosities that

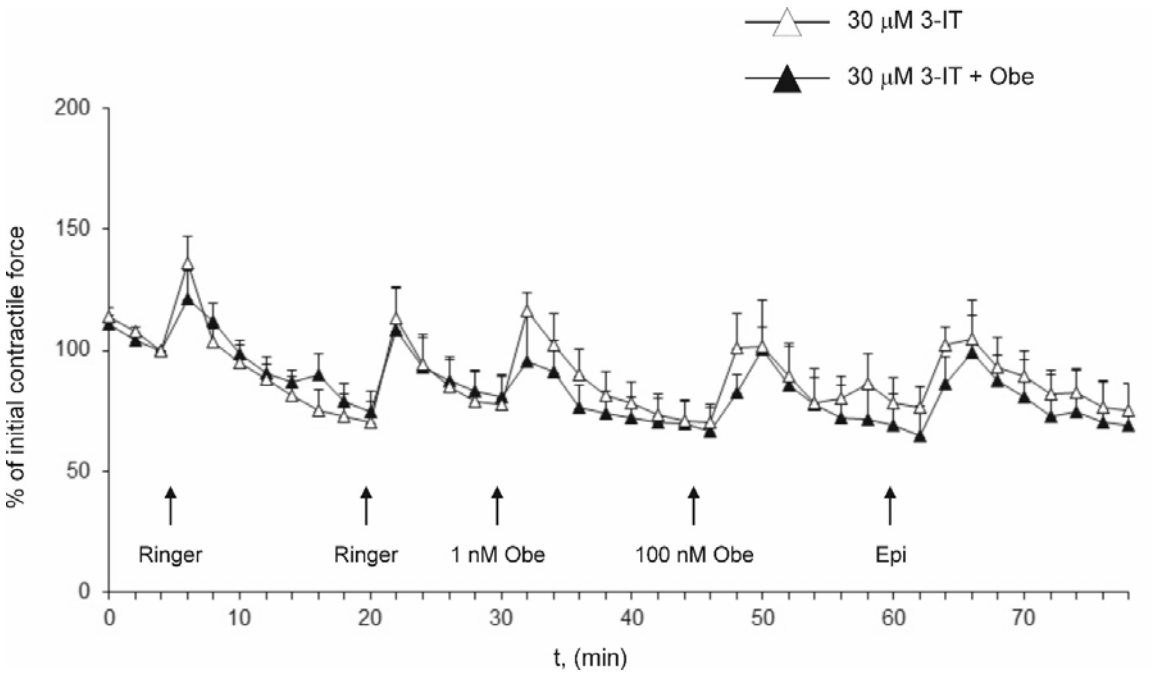


**Figure 1.** Effect of obestatin on the maximal force of contractions after treatment with 0.02 mg/g b.w. 3-IT. Time course of spontaneous frog heart contractions after 3-iodotyrosine treatment (△) is compared to that after the addition of obestatin (▲). Data are means ± SEM, n = 6. 3-IT – 3-iodotyrosine; Epi – epinephrine; Obe – obestatin.

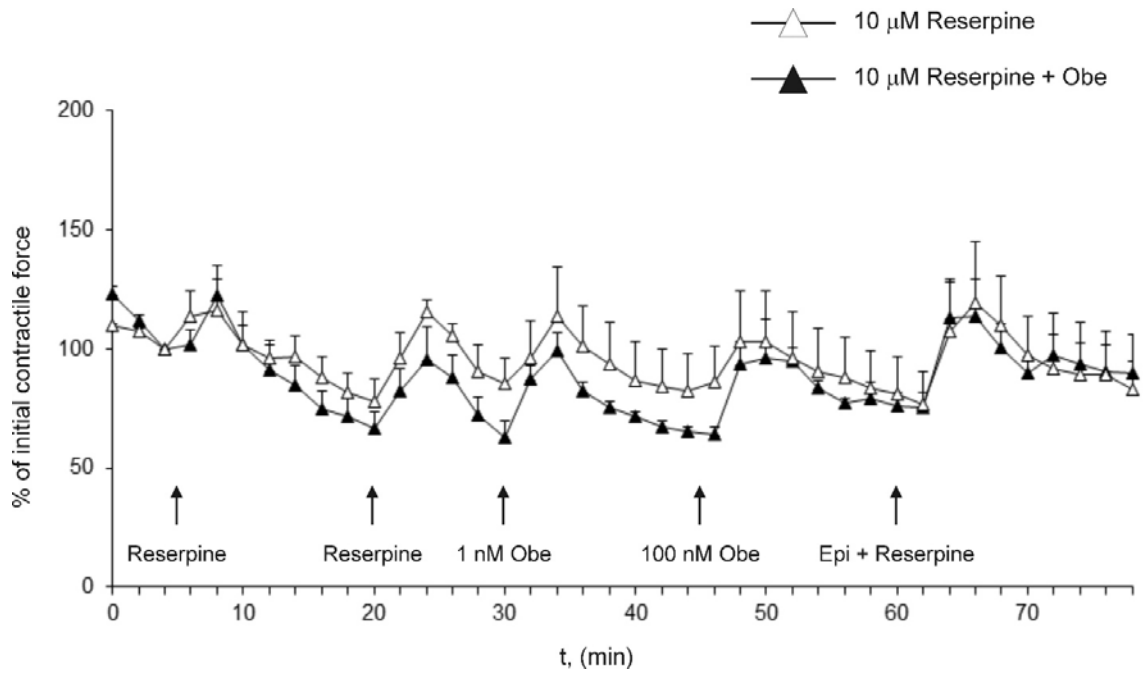


**Figure 2.** Effect of obestatin in the presence of 10 μM reserpine. Amplitude of maximal force of heart contractions in the presence of reserpine (△) was compared to that of obestatin and reserpine (▲). Data are means ± SEM, n = 6; Epi – epinephrine; Obe – obestatin.

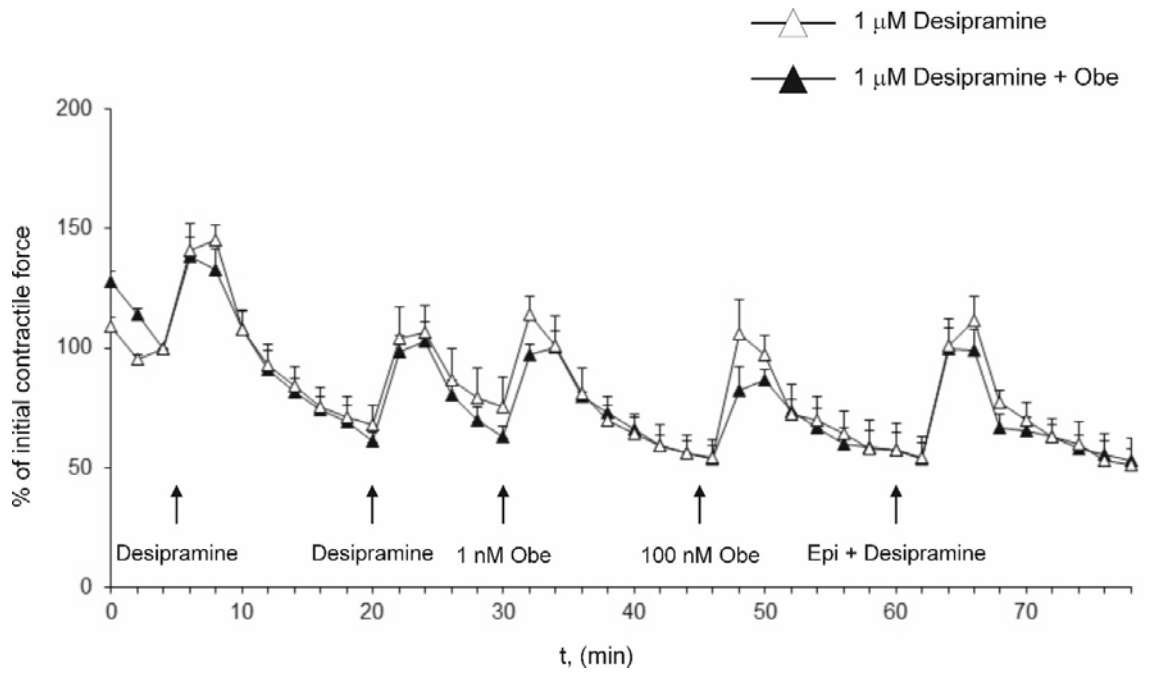


**Figure 3.** Effect of obestatin in the presence of 1 μM desipramine on the maximal force of contractions of frog heart preparation. Amplitude of heart contractions in the presence of desipramine (△) was compared to that after simultaneous application of obestatin and desipramine (▲). Data are means ± SEM, n = 6. Epi – epinephrine; Obe – obestatin.

**Table 1.** Summary data on the increase in amplitude of heart contractions after obestatin treatment. Values refers to difference between groups and * indicates statistical significance (p<0.001 - ***, p<0.01 - **, p<0.05 - *, ns – not significant).

| **Application** | **1 nM Obestatin** | | | **100 nM Obestatin** | | | **Epinephrine** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time, min** | **2nd** | **4th** | **6th** | **2nd** | **4th** | **6th** | **2nd** | **4th** | **6th** |
| Obestatin vs time control | 51% (*) | 46% (*) | 47% (*) | 62% (*) | 49% (*) | 36% (*) | 75% (**) | 49% (**) | 25% (**) |
| Desipramine + Obestatin vs time control | 32% (***) | 26% (**) | 24% (*) | 18% (*) | 18% (*) | 16% (ns) | 36% (*) | 24% (*) | 3% (ns) |

seldom come into close contact (<50 nm) with the cardiac muscle cells [20]. Thus, any observed effect is mediated only by these tissues and therefore, the *in vitro* frog heart preparation is suitable for studying the mechanism of neurotransmitters or pharmacological agents that influence the myocardium directly or indirectly *via* autonomic neurons.

Our studies show that obestatin in nanomolar concentrations (Table 1) has a significant inotropic effect on excised frog heart *via* epinephrine-sensitive mechanism [8, 9]. Obestatin may activate heart muscle contraction either upstream of adrenergic receptors by an increase of neuronal epinephrine secretion [21] or downstream in muscle tissue by a sensitization of adrenergic signaling. The hypothesis on indirect neuronal regulation of obestatin was studied using several pharmacological agents – VMAT inhibitor, norepinephrine transporter (NET) inhibitor and TH inhibitor. All of them were applied prior to obestatin treatments (1 nM and 100 nM) in order to decrease or eliminate the stimulated epinephrine secretion.

The effect on target tissue depends on the released amount of the neurotransmitter, which is the result of the intensity of two processes – secretion and removal by reuptake, chemical transformation, or diffusion. To determine the role of neurons on the inotropic effect of obestatin, we inhibited both processes. The specific inhibitors of VMAT, TH or NET were administered for emptying the epinephrine-containing secretory vesicles of sympathetic axon projections and thus reduce or even eliminate the epinephrine secretion. The data obtained show that the effect of obestatin is abolished when added in the presence of reserpine or 3-IT.

NET was blocked by desipramine to prevent the epinephrine reuptake. This treatment was expected to reduce its secretion; nevertheless, in excised frog heart the role of epinephrine reuptake is not expected to be the major process determining the capacity of its secretion. In the absence of synaptic contacts between sympathetic axons and cardiac myocytes the diffusion is of a great importance for neurotransmitter inactivation. Our results show that the presence of desipramine reduces the positive inotropic effect of obestatin by up to 50% (Table 1). These experiments enrich our knowledge on the importance of reuptake process for the general epinephrine turnover in the studied object.

Figure 4 illustrates the steps of catecholamine cycle in detail and the target molecules of used pharmacological agents.

## CONCLUSION

It is concluded that positive inotropic effect of obestatin on maximal force of contraction is mediated by the neuronal and epinephrine-dependent mechanism. This regulation most probably is achieved by increase of epinephrine secretion. The simultaneous inhibition of its reuptake could also not be excluded.

## ACKNOWLEDGEMENTS

This work was supported by grant № 80-10-108/25.03.2021 from Sofia University‘St. Kliment Ohridski’ Grant KP-06-OPR 03/18 from the Scientific Research Fund of the Ministry

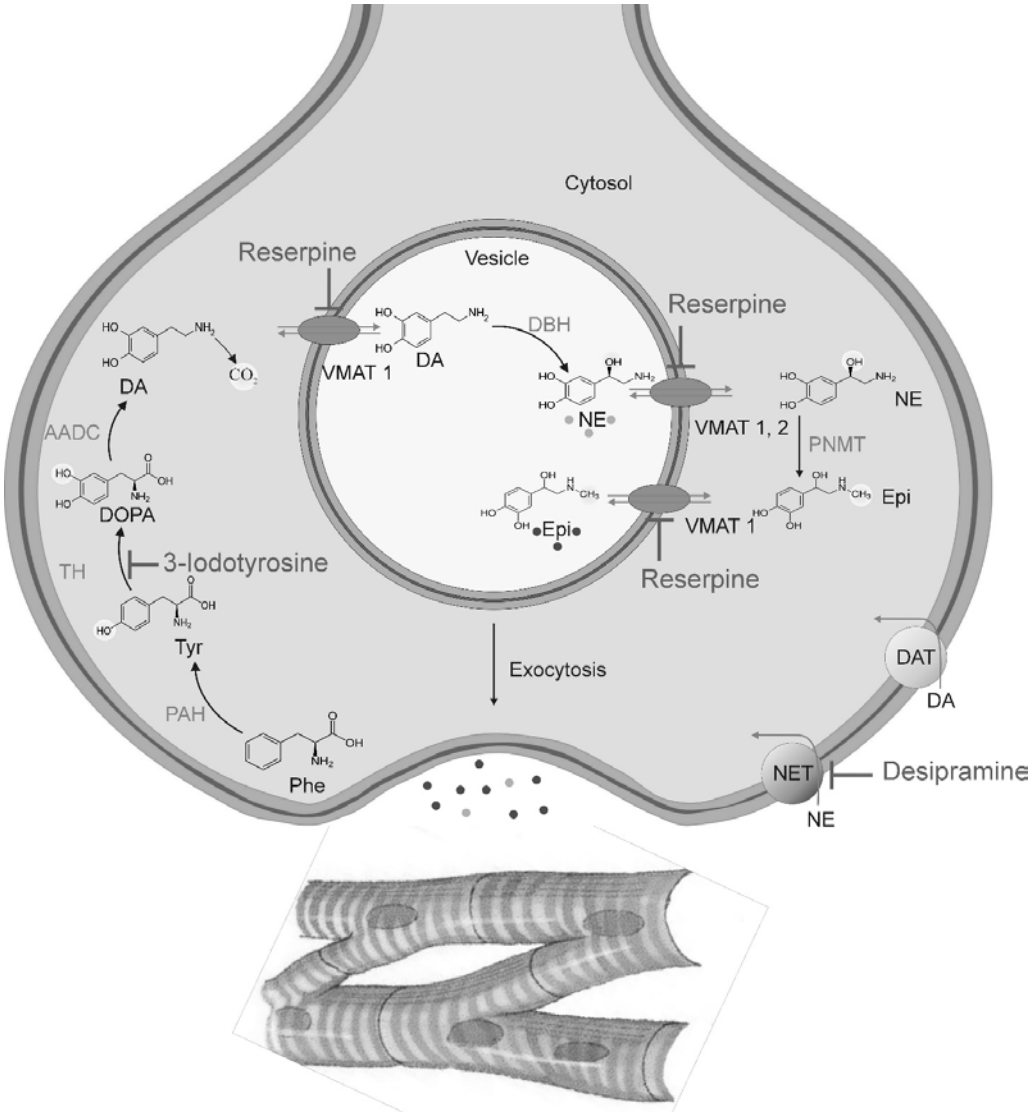


**Figure 4.** Biosynthesis of catecholamines. T-shaped arrows indicate the inhibited steps in catecholamine synthesis and the inhibitor used for this purpose.

AADC – aromatic L-amino acid decarboxylase, DA – dopamine, DBH – dopamine-β-hydroxylase, DOPA – L-3,4-dihydroxyphenylalanine, Epi – epinephrine, NE – norepinephrine, NET – norepinephrine transporter, PAH – phenylalanine-4-hydroxylase, Phe – phenylalanine, PNMT – phenylethanolamine N-methyltransferase, TH – tyrosine-3-hydroxylase, Tyr – tyrosine, VMAT – vesicular monoamine transporter.

of Education and Science of Bulgaria and BG05M2OP001-1.002-0012-C01 from Operational Programme 'Science and Education for Smart Growth' 2014-2020.

## CONFLICT OF INTEREST STATEMENT

The authors certify that they have no conflict of interest.

## REFERENCES

1. Zhang, J. V., Ren, P. G., Avsian-Kretchmer, O., Luo, C. W., Rauch, R., Klein, C. and Hsueh, A. J. W. 2005, Science, 310, 996-999.
2. Gualillo, O., Lago, F., Casanueva, F. F. and Dieguez, C. 2006, Molec. Cell Endocr., 256, 1-8.

3. Yu, A. P., Ugwu, F. N., Tam, B. T., Lee, P. H., Ma, V., Pang, S., Chow, A. S., Cheng, K. K., Lai, C. W., Wong, C. S. and Siu, P. M. 2020, Sci. Rep., 10, 5495.
4. Szentpéteri, A., Lőrincz, H., Somodi, S., Varga, V. E., Paragh, Jr. G., Seres, I., Paragh, G. and Harangi, M. 2018, Lipids Health Dis., 17, 39.
5. Aragno, M., Mastrocola, R., Ghé, C., Arnoletti, E., Bassino, E., Alloatti, G. and Muccioli, G. 2012, Cardiovasc. Diabetol., 11, 129.
6. Green, B. D. and Grieve, D. J. 2018, Peptides, 100, 249-259.
7. Alloatti, G., Arnoletti, E., Bassino, E., Penna, C., Perrelli, M. G., Ghé, C. and Muccioli, G. 2010, Am. J. Physiol. Heart Circ. Physiol., 299, H470-H481.
8. Sazdova, I., Ilieva, B., Minkov, I., Schubert, R. and Gagov, H. 2009, Open Life Sci., 4, 327-334.
9. Sazdova, I., Ilieva, B., Shkodrova, M., Milusheva, A., Chichova, M., Schubert, R., Fülüp, F., Lubomirov, L. and Gagov, H. 2013, C. R. Acad. Bulg. Sci., 66, 847-856.
10. Su, X. J., Dongb, R. X., Li, Y. P., Yangb, S. G. and Li, Z. F. 2014, Peptides, 52, 58-60.
11. Penna, C., Tullio, F., Femmin, S., Rocca, C., Angelonem T., Cerra, C., Gallo, M. P., Gesmundo, I., Fanciulli, A., Brizzi, M. F., Pagliaro, P., Alloatti, G. and Granata, R. 2017, J. Cell. Mol. Med., 21, 3670-3678.
12. Ask, J. A. 1983, Comp. Biochem. Physiol. A Comp. Physiol., 76, 543-552.
13. Fernandez-Espejo, E. and Bis-Humbert, C. 2018, Neurotoxicology, 67, 178-189.
14. Bernstein, A. I., Stout, K. A. and Miller, G. W. 2014, Neurochem Int., 73, 89-97.
15. Pimoule, C., Schoemaker, H. and Langer, S. Z. 1987, Eur. J. Pharmacol., 137, 277-280.
16. Zhou, J. 2004, Drugs Future, 29, 1235-1244.
17. Zhu, M. Y., Kim, C. H., Hwang, D. Y., Baldessarini, R. J. and Kim, K. S. 2002, J. Neurochem., 82, 146-153.
18. Hunt, C. C. and Nelson, P. G. 1965, J. Physiol., 177, 1-20.
19. Acierno, R., Gattuso, A., Cerra, M. C., Pellegrino, D., Agnisola, C. and Tota, B. 1994, Gen. Pharmac., 25, 521-526.
20. Hartzell, H. C. 1980, J. Cell Biol., 86, 6-20.
21. Ilieva, B., Gagov, H. and Sazdova, I. 2020, Curr. Top. Pharmacol., 24, 1-11.