SOFIA UNIVERSITY „ST. KLIMENT OHRIDSKI“
FACULTY OF BIOLOGY
DEPARTMENT OF ANIMAL AND HUMAN PHYSIOLOGY

Bilyana Mariyanova Ilieva

Obestatin inotropic effect on frog heart (*Pelophylax ridibundus*). Role of autonomic innervation

PhD Thesis Abstract

for acquiring the education and science degree “Doctor”
in the professional field 4.3. Biological Sciences,
scientific speciality: Animal and Human Physiology

Supervisor
Prof. Hristo Gagov PhD

Sofia 2022

ABBREVIATIONS USED

AC - adenylate cyclase
DAG- diacylglycerol
ITF – inositol triphosphate
MAO – monoamine oxidase
PK A – protein kinase A
PK C – protein kinase C
PK G – protein kinase G
FLS λ – phospholipase Cλ
cAMP – cyclic adenosine monophosphate
6 OHDA – 6-hydroxydopamine
BH4 - tetrahydrobiopterin
CAMK II – Ca/calmodulin dependent protein kinase II
ERK - extracellular signal-regulated kinase
GHS-R 1a – growth hormone secretagogue receptor 1a
GLP-1 - glucagon-like peptide 1 (GLP-1)
GLUT-4 – glucose transporter type 4
GPR39 – G protein-coupled receptor 39
LDL – low-density lipoproteins
HDL – high density lipoproteins
3IT – 3 iodo-L-tyrosine
L-dopa – L-dihydroxy phenylalanine
MAPKAP-K II - mitogen-activated protein kinase-activating protein kinase II
MSK1 – mitogen- and stress-activated protein kinase
NET – norepinephrine transporter
NO – nitric oxide
PI_3K/Akt - PI3K (phosphatidylinositol 3-kinase) and Akt (protein kinase B)
PRAK – p38 regulated protein kinase
SERCA – sarcoplasmic Ca 2+ ATPase
ROS – reactive oxygen species
VMAT 1\2 – vesicular monoamine transporters 1 and 2

INTRODUCTION

Obestatin was discovered in 2005 by Zhang et al. It is a ghrelin-related peptide that is produced by post-translational processing of the preproghrelin. As a recently identified mediator, It has attracted great interest of researchers, mainly studies of its physiological functions.

Initially, its action is associated with the activation of G-protein coupled receptor (GPR 39) and motor activity suppression of the gastrointestinal tract and food intake. Obestatin remains a controversial peptide, both in terms of nutrition and its receptor, which is still a subject of debate.

Positive effects of obestatin on glucose and lipid metabolism have been observed, suggesting an interaction with the glucagon-like peptide 1 receptor. Its role in cardiovascular control is also still unclear and controversial. Evidence to date suggests that obestatin is involved in blood pressure regulation and has beneficial effects on endothelial function. Experimental studies have shown that it has a cardioprotective effect in conditions such as ischemic heart disease.

Furthermore, obestatin exerts various cellular effects by increasing proliferation and inhibiting apoptosis and inflammation in different cell types.

Despite over 15 years of research, the obestatin influence on various organs and its mechanism of action caused a lot of discussion. This is due both to its as-yet-unknown receptor and to the eventual breakdown of its molecule into smaller fragments that are thought to have their own effects.

Similar to ghrelin, obestatin is also produced in the gastrointestinal tract, including the pancreas and adipose tissue. It exerts both local actions in peripheral tissues and distant effects at the central level, which is reason to consider it as a potential therapeutic agent. Thus, obestatin continues to be the subject of intensive scientific research.

II. MAIN OBJECTIVE AND TASKS

The main objective of the dissertation is to establish the participation of autonomic nerve endings in the obestatin effect on the contractile activity of the frog heart (*Pelophylax ridibundus*) and the mechanism of its action.

For the completion of the defined objective the dissertation defines the following tasks:

1. Explore the obestatin effect on the maximal strength of frog heart contractions after specific inhibition of catecholamine synthesis.
2. Explore the obestatin effect on the heart activity of the isolated frog heart after blocking vesicular monoamine transport.
3. Explore the obestatin effect on the maximal force of contraction of the isolated frog heart after blockade of adrenaline reuptake.
4. Examine the obestatin effect on frog heart activity after pharmacological elimination of autonomic neuron endings by *in vitro* administration of the neurotoxin 6-hydroxydopamine.
5. Examine the obestatin effect on frog heart activity after pre-injection of 6-hydroxydopamine.

III. MATERIALS AND METHODS

Experimental procedures

For the *in vitro* obestatin assays, after anesthesia and denervation, frogs were fixed in a paraffin tray. The chest cavity was opened and the pericardium was cleaned, including the blood vessels area. The heart was perfused with about 2 ml of Ringer's solution to remove blood from the aorta to avoid occlusion of the cannula by clot formation. Three ligatures were placed and tightened in the following sequence: the first - under the aortic cone was pulled down as much as possible so that part of the venous sinus remained to the preparation; the second – on the right branch of the aorta and the third – on the left branch of the aorta. Tightening of the first ligature stopped entering the blood in the heart, and then the next ligature was also tightened. A transverse incision was made about ½ of the diameter of the left branch of the aorta. The tip of the cannula was

inserted into this hole. During systole, when the aortic valve is open, it was inserted into the ventricle. The third ligature was tightened around the cannula above its extension. All vessels and tissues behind the ligatures were excised and the cannula was fixed on a tripod in a vertical position. The heart was connected to highly sensitive force-displacement transducer GRASS FT03 (Grass Instrument Co., Quincy, USA). Contractions were recorded and analiysed on a computer using interface and TENZSU (фиг. 10) software (Stocks, Sofia, Bulgaria).

The applied *in vitro* inhibitors reserpine (10μmol/l), desipramine (1μmol) and 6-hydroxydopamine (6-OHDA) (10 mmol/l) were diluted in Ringer's solution and were administered at a final volume of 200 μl into the cannula 25 minutes prior to obestatin administration. The inhibitors were also present at all subsequent administrations until the end of the experiment. 3-Iodothyrosine (3-IT) at a dose of 0.02 mg/g b.w. and with 6-OHDA at a dose of 40 μg/g b.w. was applied in vivo. Inhibitors were injected into the dorsal lymph sac one hour prior to the isolation of the hearts. At the end of each experiment, 50 μmol/l eppinefrine dissolved in Ringer's solution was introduced to a final volume of 200 μl.

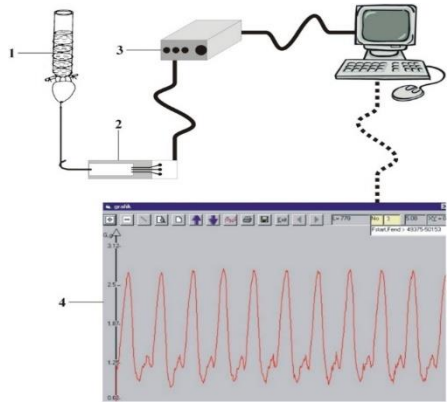

Figure 10. Experimental set-up TENZSU for recording the frog heart contractions: the abscissa represents the time, and the ordinate represents the strength of the cardiac contractions; 1 – cannula with isolated heart; 2 – sensor; 3 – analog-digital converter; 4 – original record.

Statistical analysis

The recording system amplifies 3 times the received signal. The actual force of cardiac contractions was expressed in millinewtons (mN) after threefold reduction of the recorded amplified amplitude. The obtained values showed absolute value of the force of heart contractions at the time of the study. Averaging the values for the strength of cardiac contractions in each experimental group and comparing between individual groups was impossible due to the individual characteristics of the used animals. Therefore, the strength of cardiac contractions was presented as a % of the strength measured at the 4 minute from the start of the experiment, taken as 100%.

Experiments were performed at room temperature (18-22 oC). All substances were dissolved in Ringer's solution and introduced into the cannula in a final volume of 200 µl. In the control experiments, the preparations were treated only with pure Ringer's solution at intervals corresponding to the introduction of the remaining substances for 75 min.

All data are presented as means ± SEM. The *n* refers to the number of frog heart preparations for each experimental group. Statistical significance was determined by Student's t-test for independent samples. Data with $p<0.05$ were considered statistically significant.

IV. RESULTS

Effect of obestatin on frog heart activity

In our experimental conditions, preparations of isolated frog hearts *in vitro* showed normal contractile activity with a relatively stable amplitude of cardiac contractions. The force of spontaneous contractions of the isolated heart decreased most strongly during the first few minutes, after which their decrease was insignificant during the course of the experiment. For this reason, obestatin was introduced 30 min after the start of the experiment, allowing time for adaptation and 15 min for treatment with the inhibitors administered *in vitro.*

Obestatin concentrations of 1 and 100 nM were administered at 15 min intervals. Obestatin showed an increasing statistically significant positive inotropic effect on cardiac activity of the isolated frog heart at both applied concentrations (Fig. 13).

The positive inotropic effect of obestatin was observed within about 6 min. The lowest concentration (1 nM) increased the amplitude of cardiac contractions by 65% (n=6) at 2 min after obestatin administration, by 56% at 4 min after administration, and by 28% at 6 min after obestatin treatment. At a concentration of 100 nM, the increase was 72% at 2 min after the introduction of obestatin and reached a maximum value at 4 min, but also decreased more rapidly compared to the lower concentration. Four minutes after treatment with 100 nM obestatin the increase was by 54%, and after 6 min – by 25%.

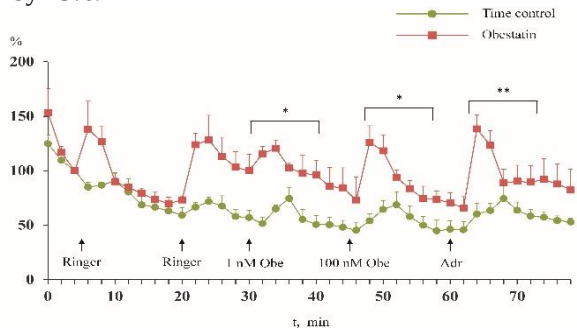

Figure 13. Effect of obestatin on the strength of cardiac contractions of a frog heart. *The effect of obestatin ( ) in in vitro conditions was compared with the control amplitudes ( ), in which the introduction of obestatin was replaced by the introduction of Ringer's solution in the same volume. *p<0.05;**p<0.01 compared to the control amplitude in Ringer's solution. Data are presented as mean ± SEM (n=6)*

Effect of obestatin on frog heart activity after 3-IT treatment

The positive inotropic effect of obestatin and the previously obtained data directed our attention to study the involvement of autonomic nerve endings in the observed effect. For this reason, a series of experiments with specific pharmacological elimination of adrenergic signaling was performed.

3-IT, a selective inhibitor of tyrosine hydroxylase, which is the rate-limiting enzyme in catecholamine biosynthesis pathway, was used. 3-IT is a physiological molecule, but at concentrations higher than its physiological serum values, causes the death of neurons that express tyrosine hydroxylase, such as dopaminergic neurons of the *substantia nigra* or noradrenergic sympathetic endings of the autonomic nervous system (Fernández-Espejo et. al. 2018). 3-IT is

found both in organs where the biochemical pathways of its biosynthesis and degradation are present - the thyroid gland (Ludgate et. al. 2011), but also in non-thyroid structures such as the gastrointestinal tract, kidneys and liver (De La Vieja et. al. 2000, Lai et. al. 2006, Ludgate et. al. 2011). Serum levels of 3-IT in frogs are not known. Therefore, we chose to inject it at a concentration (30 μM) that is about 40-fold above its physiological levels in human serum (0.69 ± 0.20 nM) (Tan et. al. 1990).

The inhibitor 3-IT was injected into the dorsal lymph sac of the frog one hour before isolation and cannulation of the heart at a dose of 0.02 mg/g body weight.

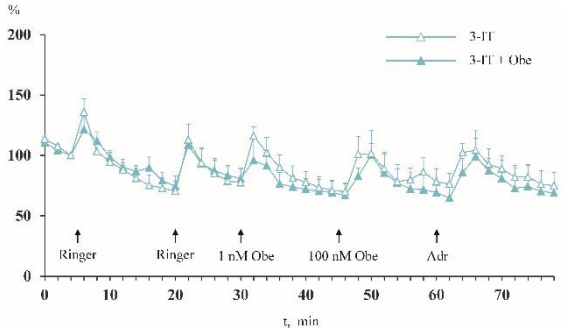

Figure 14. Effect of obestatin on the maximal force of contractions of an isolated heart preparation after pre-treatment with 3-IT 0.02 mg/g b.w: *The amplitude of cardiac contractions (△) after 3-IT pre-treatment was compared with that in the presence of obestatin (◢) after pre-treatment of 3-IT. Data are presented as means ± SEM (n=6).*

In *in vivo* treatment with this inhibitor, the first concentration of obestatin was administered 30 min after the start of the experiment. This method of administration ensures its necessary time of action and manifestation of its effect on catecholamine biosynthesis in varicose endings of sympathetic axons. 3-IT at this concentration abolished the inotropic effect of obestatin on the maximal force of contractions in both applied concentrations - 1 and 100 nM (Fig. 14). It lowered the strength of the contractions and brought them closer to control values, indicating its lasting effect on the amount of catecholamines secreted throughout the experiment.

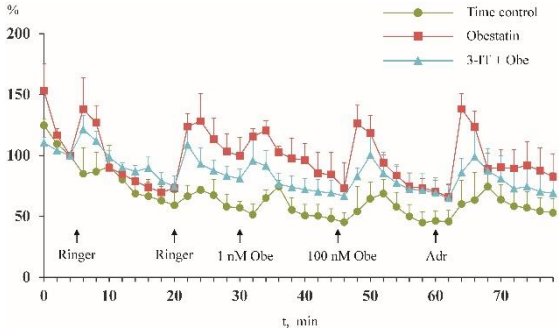

Figure 15. Comparison of the effect of obestatin in the absence and presence of the 3-IT inhibitor*. The effect of obestatin after pre-treatment of 3-IT (▲) is compare to its effect (■)in in vitro conditions. The control amplitudes of heart contractions (●) are shown for comparison. Data are presented as means ± SEM (n=6).*

In the presence of the 3-IT inhibitor, obestatin did not show a statistically significant positive inotropic effect, although the force of cardiac contractions was increased compared with that under control conditions. Such an increase in the force of cardiac contractions was also observed in the control experiments treated with the inhibitor alone. The latter result is consistent with literature data that 3-IT increases the rate of calcium release and reuptake in human cardiomyocyte cell cultures, suggesting an additional effect of 3-IT via a catecholamine-independent mechanism through direct action on cardiac muscle cells (Yang et. al. 2014).

Thus, 3-IT reduced the positive inotropic effect of obestatin on frog heart activity by 31%, 53%, and 89% relative to the pure effect of the peptide, respectively, at the 2, 4, and 6 minutes after the introduction of obestatin at a concentration 1 nM. The reduction of the effect at a concentration of 100 nM was 60%, 33% and 36% at the 2, 4 and 6 minutes of obestatin administration, respectively.

Effect of obestatin on frog heart activity in the presence of reserpine

The involvement of epinephrine storage in vesicles in the positive inotropic effect of obestatin was investigated using reserpine. Active transport of catecholamines into synaptic vesicles for their exocytosis is carried out by the vesicular monoamine transporters VMAT1 and VMAT2 (Bernstein A. I. 2014). VMAT1 (SLC18A1) is mostly expressed in the periphery, in the sympathetic autonomic nervous system, in adrenal chromaffin cells and endocrine

cells in the intestine. VMAT2 (SLC18A2) is expressed in both the peripheral (enteric nervous system, adrenal chromaffin cells, gastric endocrine cells and platelets) and central nervous system (all monoaminergic brain neurons) (Weihe et. al.1994).

Vesicular storage can be inhibited by substances such as reserpine. These substances reduce amines in neurons by blocking their entering into vesicles. They also block the re-entering of dopamine into vesicles after its reuptake from the synaptic cleft. Dopamine in the terminal that is not included in the vesicle is deaminated by the enzyme monoamine oxidase (MAO), which is located in the nerve terminal or bound to the outer side of the membrane of neuronal mitochondria (Webster 2001). Dopamine in the terminals of noradrenergic neurons is transported into vesicles, where the vesicular enzyme dopamine-β-hydroxylase hydroxylates the β-carbon atom and thereby converts dopamine to norepinephrine. Therefore, in neurons with blocked deposition of dopamine in vesicles, secretion of dopamine and its metabolites norepinephrine and adrenaline does not occur (Webster 2001). Reserpine is an irreversible and nonspecific inhibitor of VMAT1 and 2 (Liu et. al. 1992, Erickson et. al. 1996). By blocking the transphosphatase that maintains the proton gradient, it gradually depletes the mediator from neuronal vesicles (Webster 2001).

Reserpine was administered at a concentration of 10 μmol/l. It was introduced into the cannula along with Ringer's solution 25 min before obestatin treatment and at each subsequent introduction till the end of the experiment.

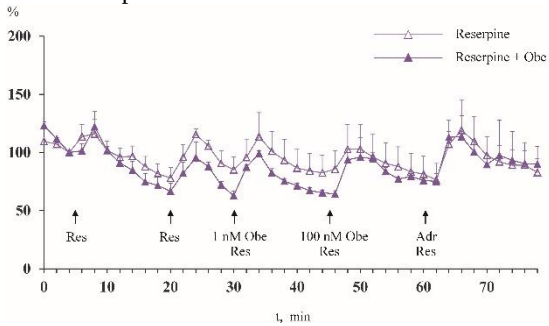

Figure 16. Effect of obestatin on the maximal force of contractions in the presence of reserpine: *The effect of obestatin (▲) on the contractions of the frog heart was compared with control amplitudes in the*

presence of reserpine (△) in in vitro conditions. Data are presented as mean ± SEM (n=6)

As shown in fig. 16, the effect of obestatin was completely abolished by reserpine, which lowered the amplitude of cardiac contractions below control values. The positive inotropic effect of obestatin was not observed at any of the applied concentrations

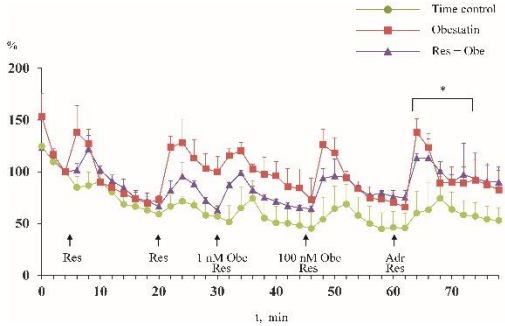

Figure 17. Comparison of the effect of obestatin in the absence and presence of the inhibitor reserpine*. The effect of obestatin in the presence of 10 µmol/l reserpine (▲) is compare to its effect (■) in vitro conditions and relative to control amplitudes (●). **p<0.01 and *p<0.05 relative to the control amplitude in Ringer's solution. Data are presented as mean ± SEM (n=6).*

Compared to its pure effect, obestatin in the presence of reserpine did not show a statistically significant positive inotropic effect, although the force of cardiac contractions was increased compared to that of the control (Fig. 17). The combination of obestatin with reserpine statistically significantly increased the force of heart contractions compared to control amplitudes. This increase in the force of cardiac contractions was also observed in the control experiments that were treated with the inhibitor alone. This could be due to the reserpine-induced increase in the cytosolic concentration of adrenaline which causes its binding to the reuptake transporter, but on its cytoplasmic side (Webster 2001). Therefore, the direction of its transport is out of the cell not inward (reverse uptake), which activates postsynaptic adrenoceptors and thus causes the increased force of cardiac contractions that we observe. This form of mediator release is called retrotransport and occurs independently of neuronal excitability or intracellular calcium concentration. (Webster 2001).

Reserpine reduced the inotropic effect of obestatin by 45%, 39%, and 71% relative to the pure effect of the peptide, respectively, at the 2, 4, and 6 minute of obestatin administration at a concentration of 1 nM. The reduction of the effect at a concentration of 100 nM was 44%, 41% and 4% at the 2, 4 and 6 minutes of administration, respectively.

The adrenaline, added at the end of the experiment in a concentration of 50 μmol/l, led to a short-term but statistically significant increase in the force of heart contractions compared to the control values (Fig. 17).

Effect of obestatin on frog heart activity in the presence of desipramine

The importance of the reuptake mechanism of synaptically released neurotransmitters for the obestatin effect was explored with desipramine. The transporter controlling the synaptic concentration of catecholamines is a protein that transports them back through a reuptake mechanism in the presynaptic membranes of the corresponding neurons (Webster 2001). Norepinephrine transport occurs by co-transport of Cl- and Na+ ions. Binding of Na+ and norepinephrine to the transporter on its extracellular side leads to conformational changes that open an inward channel in the transporter. This allows the passage of norepinephrine from the extracellular space into the cytosol of the neuron (Webster 2001). Many intracellular mediators can modify the substrate affinity of the transporter by phosphorylation or glycosylation and thus affect its function (Bönisch et. al. 1998).

The tricyclic antidepressant desipramine is a selective inhibitor of the noradrenaline transporter responsible for the neuronal uptake of noradrenaline (Zhou et. al. 2004, Zhu et. al. 2002). In the frog heart, adrenaline, not norepinephrine, is the sympathetic neurotransmitter (Angelakos et. al. 1965). Desipramine has been found to bind with the same affinity to the adrenaline transporter, blocking the reuptake (return) of adrenaline (Pimoule et. al. 1987). It was introduced at a concentration of 1 μmol/l into the cannula in the same manner as described for the reserpine studies.

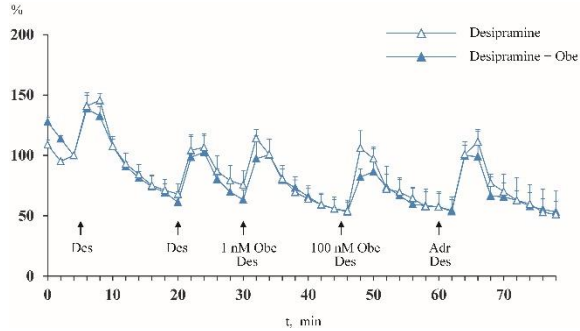

Figure 18. Effect of obestatin on the maximal force of contractions in the presence of desipramine: *The effect of obestatin (◢) on the force of heart contractions was compared with control values in the presence of desipramine (△) in in vitro conditions. Data are presented as mean ± SEM (n=6)*

In the presence of desipramine, the force of cardiac contractions remained comparable to that under control conditions. The positive inotropic effect of obestatin at both applied concentrations (1 nM and 100 nM) was completely blocked in the presence of 1 μmol/l desipramine (Fig. 18).

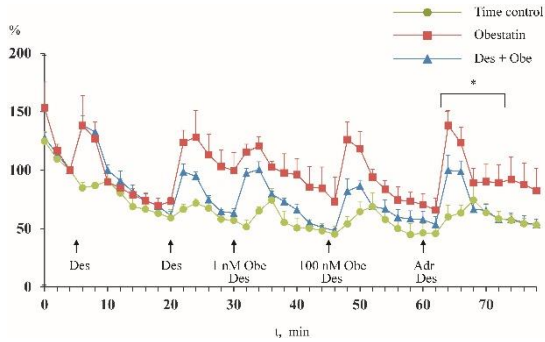

Figure 19. Comparison of the effect of obestatin in the absence and presence of the inhibitor desipramine*. The effect of obestatin in the presence of 1 μmol/l desipramine (◢) is compare to its effect (■) in in vitro conditions. The control amplitudes (●) are given for comparison. ***p<0.001 and *p<0.05 compared to the control amplitude in Ringer's solution. Data are presented as means ± SEM (n=6).*

Compared to its pure effect, obestatin in the presence of desipramine showed a weaker but statistically significant positive

inotropic effect to control amplitudes (Fig. 19). Desipramine reduced the inotropic effect of obestatin by 29%, 36%, and 78% relative to the pure effect of the peptide, respectively, at the 2, 4, and 6 minute of obestatin administration at a concentration of 1 nM. The reduction of the effect at a concentration of 100 nM was 61%, 57% and 80% at the 2, 4 and 6 minutes of administration, respectively (Fig. 19). The combined effect of obestatin and desipramine showed a statistically significant inotropic effect relative to control values. These results are probably due to the desipramine-induced prolonged action of adrenaline at the synapse, which is also seen in the desipramine control studies (Fig. 18, ◿ **).**

Exogenously added adrenaline increases the amplitude of heart contractions in a statistically reliable manner compared to the control ones, in which Ringer's solution was added to the corresponding input (Fig. 19).

Effect of obestatin on frog heart activity in the presence of the neurotoxin 6-OHDA

The positive inotropic effect of obestatin was also explored after pharmacological elimination of sympathetic endings preserved in the myocardial layer consisting of muscle cells arranged in bundles.

Therefore, the neurotoxic 6-OHDA was used. It is a neurotoxin that enters as a "false mediator" via the catecholamine reuptake system in catecholaminergic neurons. Within minutes, it is oxidized to several cytotoxic substances, generating free radicals and destroying dopaminergic neurons through oxidative stress and subsequent apoptosis. Additionally, 6-OHDA causes hyperpolarization of the mitochondrial membrane, which suppresses mitochondrial respiratory chain complexes (Endepols et. al. 2004).

Two series of experiments were performed with 6-OHDA. In one, it was added in the cannula and in the other, it was injected into the dorsal lymph sac one hour before heart isolation and cannulation. 6-OHDA was dissolved immediately prior to injection. To prevent its oxidation, 0.2 mg/ml ascorbic acid was added as an antioxidant.

In the first series of experiments, 6-OHDA at a concentration of 10 mmol/l with added antioxidant was introduced into the cannula of the isolated heart 25 min before the administration of the lowest concentration of obestatin (Fig. 20).

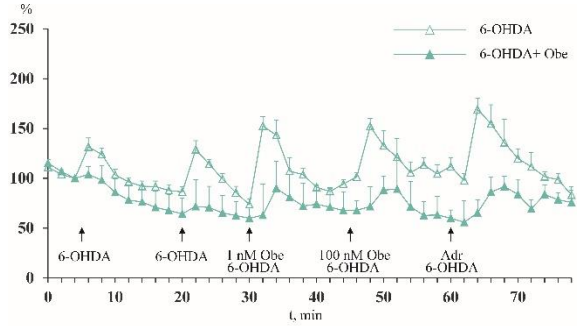

Figure 20. Effect of obestatin in the presence of 6-OHDA (10 mmol/l) on the maximal force of contractions of a frog heart in vitro. *The effect of obestatin (◢) on heart contractions was compared with control amplitudes after in vitro administration of 6-OHDA (△). Data are presented as mean ± SEM (n=6).*

In the presence of the neurotoxin 6-OHDA, obestatin did not show a positive inotropic effect. At both applied concentrations of obestatin, the force of cardiac contractions was below control values (Fig. 20) and only slightly increased relative to controls treated at the same intervals with pure Ringer's solution (Fig. 21).

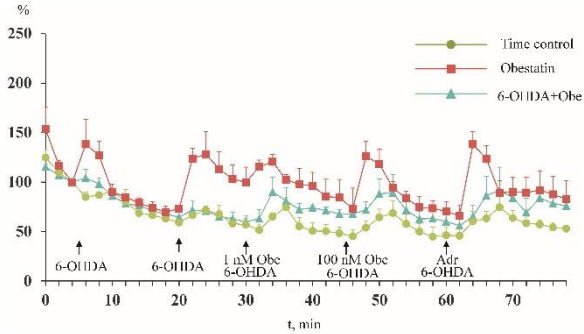

Figure 21. Effect of exogenously added adrenaline on the maximal force of contractions of an isolated frog heart preparation in the presence of the neurotoxin 6-OHDA*. The effect of adrenaline in the presence of 10 mmol/l 6-OHDA (◢) was compared with its effect after introduction of obestatin (■) in in vitro conditions and that in the control amplitudes (●). Data are presented as means ± SEM (n=6).*

To ensure sufficient time for action on adrenergic endings, we also performed a series of experiments in which a solution with 6-

OHDA (40 µg/g b.w.) was injected into the dorsal lymph sac of the frog one hour before the start of the experiment. At 30 minutes from the beginning of the *in vitro* experiment, obestatin was introduced sequentially, in both concentrations.

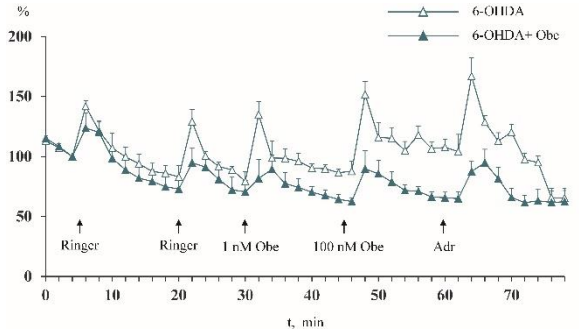

Figure 22. Effect of obestatin on the maximal force of contractions an isolated heart preparation after pre-treatment with 40 µg/g 6-OHDA*: Amplitude of cardiac contractions* (△) *after 6-OHDA pretreatment was compared with that in the presence of obestatin* (▲) *after pre-treatment with 6-OHDA. Data are presented as means ± SEM (n=6).*

In this series of experiments, obestatin did not show a positive inotropic effect. The force of heart contractions in both applied concentrations of obestatin was lower than control values (Fig. 22) and slightly increased compared to that of controls conditions (Fig. 23).

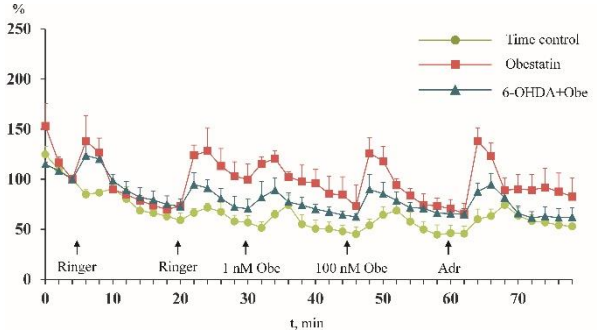

Figure 23. Effect of adrenergic mediation on the maximal force of contractions on isolated heart preparation after pre-treatment of 40 µg/g 6-OHDA. *The effect of adrenaline after injection of 6-OHDA (◢) was*

compared with its effect after added of obestatin (■) in in vitro conditions and that under control conditions (●). Data are presented as means ± SEM (n=6).

As can be seen in Figures 20 and 22, the amplitudes of the control heart contractions, both *in vitro* treatment and pre-treatment with 6-OHDA greatly exceeded those in the presence of obestatin. Moreover, the increases observed always with the application of the solutions were particularly pronounced when 6-OHDA was introduced, and significantly reduced in the presence of obestatin. This suggests an intrinsic, nonspecific effect of 6-OHDA on cardiac cells that is abolished in the presence of obestatin.

The summary data for the change in the maximal force of contractions due to exogenously added adrenaline at the end of each experiment after treatment with obestatin and the inhibitors compared to the control are presented in the table. 3.

Table 3

Summary of amplitude changes after addition of adrenaline at the end of each experiment*. Data refer to the difference between control values and those of the respective groups of experiments, and * indicates statistical significance (p<0.001 - ***, p<0.01 - **, p<0.05 - *)*

	Exogenous adrenaline		
	2-min	4-min	6-min
Obestatin (Fig.13)	78% (**)	60% (**)	15% (**)
Obe+ 3-IT (Fig. 15)	26%	36%	13%
Obe+Reserpine (Fig.17)	53% (*)	50% (*)	26% (*)
Obe+Desipramine (Fig.19)	40% (*)	36% (*)	-11%
Obe+6-OHDA (*in vitro*) (Fig. 21)	6%	23%	18%
Obe+6-OHDA (pre-treatment) (Fig. 23)	28%	32%	8%

Exogenous adrenaline added at the end of each experiment caused a strong but transitional (for about 4 minutes) increase in the force of heart contractions, compared to controls in which only Ringer's solution was added. It can be seen from Table 3 that the

increase in the corresponding minutes after the introduction of adrenaline was most significant in the experiments with obestatin and less so in the experiments with added inhibitor. The force of heart contractions as a result of the added obestatin remained high and by the end of the experiment did not fall to the control amplitudes (Fig. 13), which suggests a long-term stimulation of β1-adrenoceptors. This stimulation was induced indirectly, initially by the twice added obestatin and finally by the adrenaline itself at the end of the experiment. Since in the presence of inhibitors the effect of obestatin is suppressed, at the end of these experiments the increase in the force of heart contractions caused directly by the added adrenaline is seen, without accumulation of its effect on β1-adrenoceptors. Catecholamine-dependently activated β1-adrenoceptors have been found to sustainably stimulate the synthesis of secondary mediators (cAMP) in primary cardiomyocytes and neurons (Fu et. al. 2014). This shows that the physiological effect of adrenaline in the studied object can continue long after its removal from the synaptic cleft due to the activity of intracellular reactions that caused the synthesis of this second mediator.

V. DISCUSSION

Our results show that obestatin has a strong positive inotropic effect (Fig. 13). From the data, it is clear that this effect is realized through activation mainly of β-adrenoceptors. This is confirmed by data in which the effect of obestatin was partially blocked by prazosin (selective α1-receptor blocker) and completely by propranolol (β-adrenoceptor blocker) (Sazdova et. al. 2009). At the same time, administration of inhibitors that are not part of the intracellular signaling chain of adrenoceptors also abolish its effect (Sazdova et. al. 2009). This suggests that cardiomyocytes are not the target tissue for the effect of obestatin.

The other potential tissue through which the effect of obestatin could be realized is nerve endings. They can be stimulated to release a mediator that exerts the inotropic effect on the heart. The obtained data show that the studied peptide has a strong dose-dependent effect. It is most pronounced at the last two concentrations (100 and 1000 nM), which were applied during the last 30 minutes of the experiment (Sazdova et. al. 2009). This would be possible only if the synthesis of new mediator molecules achieve in the synaptic endings.

Our results show that inhibition of tyrosine hydroxylase, the key enzyme of the catecholamine synthesis pathway, completely suppresses the effect of obestatin (Fig. 14). Dopamine, norepinephrine, and adrenaline contain a catechol ring (a 6-carbon ring with two adjacent hydroxyl groups) and an amino group, so these amines are called catecholamines. Depending on the enzymes present in the ending, any one of these catecholamines can be released. The first two steps of their synthesis are common to all catecholamines. Their synthetic pathway starts from the amino acid tyrosine (fig. 24).

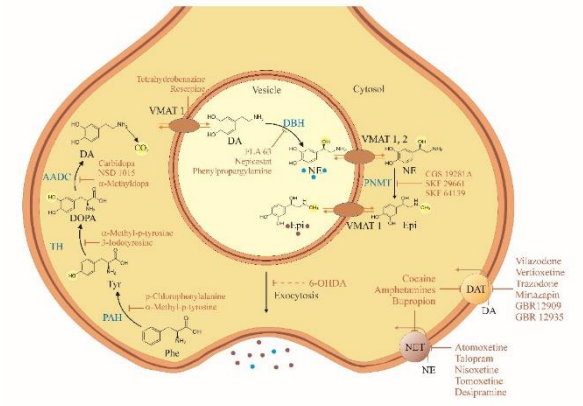

Figure 24. Biosynthetic pathway of catecholamines: *AADC – aromatic L-amino acid decarboxylase; DA – dopamine; DAT – dopamine transporter; DBH – dopamine-β-hydroxylase; DOPA – L-3,4-dihydroxyphenylalanine; Epi – epinephrine (adrenaline); NE – norepinephrine; NET – norepinephrine transporter; RAN – phenylalanine-4-hydroxylase; Phe – phenylalanine; PNMT – phenylethanolamine N-methyltransferase; TN – tyrosine-3-hydroxylase; Tyr – tyrosine; VMAT – vesicular monoamine transporter.*

In axonal endings, the cytosolic enzyme tyrosine hydroxylase converts tyrosine to L-dopa. This is the rate-limiting reaction in the synthesis, requiring the presence of molecular oxygen and Fe^{2+} as well as tetrahydrobiopterin (BH4) (Smith 2002).

As a key control unit in the synthesis of catecholamines, tyrosine hydroxylase is a subject to various regulatory mechanisms. The enzyme has a multidomain structure, with a regulatory domain (R) of 100-150 amino acid residues at the N-terminus, followed by a catalytic domain (C) of about 330 residues and a C-terminal domain

of about 20 amino acids. Domain R is involved in regulating the activity of the enzyme. Modes of regulation include phosphorylation by multiple kinases at 4 different serine residues and dephosphorylation by 2 phosphatases. The enzyme is inhibited by negative feedback from the end products of the synthesis - catecholamines (Daubner et. al. 2011).

Dopamine, norepinephrine, and adrenaline inhibit tyrosine hydroxylase activity via a negative feedback pathway. They bind in the active site of the enzyme, which overlaps with tetrahydrobiopterin, and as a result, the conformation of the R-domain changes, which prevents the entry of new substrate molecules into the active site of the enzyme (Daubner et. al. 2011). Catecholamines bound to tyrosine hydroxylase reduce its activity by about 15% (Gordon et. al. 2008). They are released from the active site if the enzyme is phosphorylated. Phosphorylation of tyrosine hydroxylase by PK A at Ser40 decreases its affinity for catecholamines about 300-fold (Ramsey& Fitzpatrick 1998) A second, lower-affinity binding site for catecholamines is believed to exist for tyrosine hydroxylase, resulting in an additional 10-fold decrease in its activity. It is not inhibited by phosphorylation at Ser40, and binding and release from tyrosine hydroxylase is dependent on catecholamine concentration (Dickson & Briggs 2013). This suggests that this binding site has the ability to alter tyrosine hydroxylase activity in response to changing catecholamine concentrations in the cytosol. This site may be the primary control mechanism under basal or tonic conditions of activation of catecholaminergic neurons. Under conditions of high activity in these neurons, the low-affinity site of both unphosphorylated and phosphorylated tyrosine hydroxylase would be saturated, and phosphorylation of the enzyme would be the primary mechanism for regulating tyrosine hydroxylase activity. The complexity of tyrosine hydroxylase regulation by feedback inhibition and phosphorylation may reflect the different requirements for catecholamine synthesis in different tissues and under different conditions. Additionally, phosphorylation of Ser19 and Ser31 can increase the rate of Ser40 phosphorylation, with Ser19 increasing the rate about threefold and Ser31 increasing the rate about ninefold (Lehmann et. al. 2006, Toska et. al. 2002). This increase in phosphorylation was not observed with the catecholamine-bound enzyme, but only with the catecholamine-free tyrosine hydroxylase. This suggests that this

hierarchical phosphorylation does not play a role in the initial activation of tyrosine hydroxylase, but serves to maintain its activity at a higher level after the activation phase. Phosphorylation of Ser19 and Ser31 has been shown to enhance tyrosine hydroxylase activation in situ (Lehmann et. al. 2006).

Consistent with the above literature data, previously obtained results show that obestatin affects the neuron by activating both PK A and ERK1/2 (Sazdova et. al. 2009), which provides multisteps phosphorylation of tyrosine hydroxylase. This leads to an increase in catecholamine synthesis and to the release of adrenaline from sympathetic nerve endings and to the observed positive inotropic effect of obestatin. The used 3-IT inhibitor of tyrosine hydroxylase (fig. 14) completely abolishes the effect of obestatin by preventing the synthesis of adrenaline, which mediates this effect.

The other potentially important step in the synthesis of catecholamines and, respectively, for the effect of obestatin, is the transport of dopamine into the vesicles. Dopamine in the terminal which is not incorporated into the vesicle is degraded by MAO, that reduces the amount of catecholamines released from the terminal (Webster 2001). Furthermore, many studies in both in vitro and animal models have shown that dysregulated cytoplasmic levels of dopamine are neurotoxic (Hastings et. al. 1996, Asanuma et. al. 2003). In vitro experiments suggest that the vulnerability of dopamine neurons in Parkinson's disease may be due to cytosolic dopamine (Mosharov et. al. 2009). Moreover, in non-dopaminergic neurons of mice expressing dopamine transporter (DAT) but lacking VMAT2, dopamine reuptake occurs but is not stored in vesicles. This leads to motor deficits and severe neurodegeneration, accumulation of α-synuclein accompanied by markers of increased dopamine oxidation (Bernstein et. al. 2014).

Norepinephrine, like dopamine, is stored in large vesicles that accumulate in varicosities (Smith 2002). These vesicles contain ATP, proteins and bivalent metal ions. The concentration of noradrenaline in the vesicles is thought to be in the range of 0.1–0.2 M, which means that it exceeds its concentration in the cytosol by 10,000 to 100,000 times. This concentration gradient moves norepinephrine out into the cytoplasm. Vesicular accumulation of norepinephrine is possible due to secondary active transport via vesicular monoamine transporter (VMAT). Vesicular monoamine transporters are essential for proper monoaminergic

neurotransmission, which requires storage of the mediator in synaptic vesicles via VMAT for subsequent Ca^{2+}-stimulated exocytosis (Sudhof 2004). The main function of these transporters is to accumulate enough quantity of the mediator to be ready for release upon a signal. They also protect the neuron from the potentially toxic effects of excess cytoplasmic noradrenaline and maintain a concentration gradient facilitating noradrenaline reuptake from the synapse (Webster 2001). The entry of norepinephrine into the vesicle depends on the electrochemical gradient created by the excess of protons on the inner side of the vesicular membrane. This gradient is maintained by an ATP-dependent vesicular proton ATPase. The transfer of one norepinephrine molecule into the vesicle by the transporter is accompanied by the release of two H^+ ions (Parsons 2000). Binding or transfer of the first H^+ ion is thought to increase the affinity of the transporter for norepinephrine, and binding of the second H+ ion triggers transfer of the mediator (Webster 2001).

Our results show that inhibition of VMAT by reserpine (Fig. 16) abolishes the positive inotropic effect of obestatin on cardiomyocytes. The results lead us to suppose that obestatin activates the biosynthesis of catecholamines (in the case of adrenaline) in the nerve terminal by phosphorylating tyrosine hydroxylase. Blocking the entry of the formed dopamine into the vesicles increases its cytosolic concentration. Adrenaline entering the terminal as a result of reuptake from the synapse also accumulates in the cytosol. When neurons are stimulated, the release of catecholamines increases, but their reuptake from the synapse also increases. Although part of this mediator is metabolized by MAO and another part passes into the vesicles, a transient increase in its concentration is observed in the cytosol, leading to more intense substrate inhibition of tyrosine hydroxylase (Webster 2001). In the case when VMAT is inhibited, reuptake adrenaline also accumulates in the cytosol and its concentration there increases. Increased levels of end products of catecholamine biosynthesis led to enhanced tyrosine hydroxylase inhibition and the lack of effect of obestatin that we observed in the presence of reserpine.

Catecholamines released in the synaptic space are transported back into the neuron through a reuptake mechanism on the presynaptic membranes of the respective neurons. The transporter controlling their synaptic concentration is a protein of 619 amino acids with 12 hydrophobic membrane-bound domains and N- and C-

termini on the cytoplasmic side (Webster 2001). The noradrenaline transporter (NET) is located in the presynaptic plasma membrane of noradrenergic neurons, where it takes up synaptically released noradrenaline. Therefore, NET serves as a major mechanism for inactivating noradrenergic signaling (Zhou 2004). Only one NET protein has been identified, suggesting that any change in its expression can significantly alter norepinephrine neurotransmission (Zhu et. al. 2002). Transport of norepinephrine occurs by co-transport of Cl^- and Na^+, therefore the transporter is classified as a family of Cl^-/Na^+ transporters. Binding of Na^+ and norepinephrine to the transporter on its extracellular side induces conformational changes that open an internal channel "gate" in the transporter. This allows noradrenaline to pass from the extracellular space into the cytosol of the neuron (Webster 2001). This reuptake mechanism is referred to as neuronal uptake 1 (uptake 1) (Zhou 2004). In the central nervous system, NET inhibition significantly reduces sympathetic activity, possibly through α2-adrenoceptor-mediated mechanisms (Schroeder& Jordan 2011). In glial cells and the periphery, there is also a second mechanism, termed extraneuronal uptake 2 (Webster 2001), in which released norepinephrine is taken up by other tissues. A significant, although smaller, portion of norepinephrine taken up via the uptake 2 system is metabolized by catechol-O-methyltransferase. In the rat heart, for example, this process results in the transport of norepinephrine and other catecholamines into cardiac muscle cells (Farnebo & Malmfors 1969). Organic cation transporter 3 (OCT3) has been identified as a transporter mediating the extraneuronal uptake of norepinephrine (Schroeder & Jordan 2011).

The mammalian reuptake mechanism is most effective for norepinephrine, whereas uptake in the frog myocardium shows a preference for adrenaline after blocking this mechanism by cocaine (Stene-Larsen et. al. 1978). Conversely, the extraneuronal uptake mechanism has the highest affinity for the predominant catecholamine in the adrenal glands – adrenaline in mammals (Holzbauer & Sharman 1972) and norepinephrine in amphibians (Stene-Larsen et. al. 1978).

Substances such as desipramine block the transport of norepinephrine across the NET and lead to an increase in the synaptic concentration of the neurotransmitters (Zhou 2004). This activates presynaptic α_2-adrenoceptors, which is a Gi protein-coupled receptor

reduces the level of cAMP. This leads to a decrease in the release of norepinephrine from the presynaptic terminal (Webster 2001). Furthermore, a decrease in the intracellular concentration of cAMP indirectly decreases the entry of calcium ions into the terminal and increases K^+ conductance, which reduces the excitability of the neuron (Starke 1987). These presynaptic autoreceptors play an important role in providing sufficient neurotransmitter to maintain stores and prevent overstimulation of postsynaptic cells (Webster 2001). In the presence of desipramine, the positive inotropic effect of obestatin was completely suppressed (Fig. 18) probably due to an inability to restore the stores after the initial neuromediation.

In addition, the process of neuronal uptake is important for the entry of so-called "false mediators" into adrenergic neurons (Kopin 1968, Thoenen 1969). One of these compounds is 6-OHDA. It accumulates in adrenergic neurons, both in the periphery and in the CNS, but has the property of causing cellular damage leading to complete degeneration of adrenergic nerve endings (Iversen 1971).

Cell death induced by 6-OHDA is caused by reactive oxygen species (ROS) derived from the autoxidation of 6-OHDA, as well as by a direct effect on the mitochondrial respiratory chain (Rodriguez-Pallares et. al. 2007). The molecular mechanism underlying 6-OHDA neurotoxicity is thought to be the formation of ROS (Soto-Otero et. al. 2000). 6-OHDA has also been found to act as an inhibitor of the NADH dehydrogenase complex (complex I) of the mitochondrial respiratory chain (Glinka et. al. 1996, 1997). The two mechanisms are thought to be biochemically independent, but in vivo they act synergistically (Glinka et. al. 1997). Studies indicate that, in addition to the mentioned mechanisms, extracellular ROS generation by autoxidation of 6-OHDA also occurs. It also plays an important role in the death of dopamine neurons in the CNS (Hanrott et. al. 2006) and underlies the pathogenesis of Parkinson's disease (Block& Hong 2005; Hald & Lotharius 2005).

6-OHDA is oxidized to paraquinone, which in turn produces indoline and indole derivatives that can form covalent bonds with nucleophilic groups of macromolecules (Siggins & Forman 1973). In addition to p-quinone, the oxidative conversion of 6-OHDA also releases hydrogen peroxide in vitro (Heikkila & Cohen 1971) and in vivo (Heikkila & Cohen 1972), which is also responsible for the toxicity of 6-OHDA. In neuron-like catecholaminergic cell cultures, the adrenal pheochromocytoma cell line PC12, cell viability was

found to be significantly reduced by 6-OHDA at a concentration greater than 12.5 μM. Furthermore, it was found that 50 μM hydrogen peroxide and 103 μM p-quinone were released from 100 μM 6-OHDA (Saito et. al. 2007).

The formation of ROS causes oxidative stress that leads to apoptosis in neural and other cell types (Maritim et. al. 2003). In addition to oxidative stress, 6-OHDA also induces an endoplasmic reticulum stress response (Ryu et al. 2002; Holtz & O'Malley 2003), which is thought to be involved in the pathophysiology of Parkinson's disease. As an endogenous store for calcium ions, the endoplasmic reticulum is vital for intracellular calcium regulation. Calcium content in the lumen of the endoplasmic reticulum and in the cytosol is very important for mitochondrial function and cell vitality (Michel et. al. 2013). 6-OHDA significantly increased the cytosolic concentration of calcium ions in dopamine neurons (Qu et. al. 2014). A ryanodine calcium channel in the endoplasmic reticulum is thought to play an important role in this 6-OHDA-induced disturbance of calcium homeostasis (Huang et. al. 2017). In PC12 cell cultures, 6-OHDA leads to an increase in intracellular cAMP and Ca^{2+} levels via L-type Ca^{2+} channels and activation of the Ca^{2+}→CaMKII → adenylate cyclase → cAMP signaling chain. The resulting high levels of cAMP lead to a process of cell death (Jin et. al. 2010; Park et. al. 2013). In an attempt to determine which apoptotic pathways are activated, several studies have indicated the involvement of the mitochondrial caspase cascade in 6-OHDA-induced apoptosis. 6-OHDA induces caspase 3-dependent cleavage of pro-apoptotic protein kinase Cδ (PKCδ) (Hanrott et. al. 2006). The site of cleavage by caspase 3 is between the regulatory and catalytic domains, leading to activation of the latter (Ghayur et. al. 1996). PK C is a redox-sensitive kinase and is activated in response to oxidative stress (Kaul et. al. 2005) and appears to be a key mediator in 6-OHDA-induced cell death (Hanrott et. al. 2006). It has been shown in PC12 cells that mitochondrial dysfunction initiated by 6-OHDA induces the release of cytochrome *c*, which causes the subsequent activation of caspase-9 with formation of the apoptosome in the presence of ATP and activates caspase-3 (Hanrott et. al. 2006, Chen et. al. 2006). Caspase 3 activation occurs 6 hours after 6-OHDA administration (Saito et. al. 2007). The release of cytochrome *c* from mitochondria into the cytosol is required for the assembly of the apoptosome and therefore for the activation of the caspase cascade in

the intrinsic pathway of apoptosis (Wang 2001). Cytochrome *c* could be detected 30 min after exposure to 6-OHDA (Saito et. al. 2007). In the same study, hydrogen peroxide generated by 6-OHDA was found to induce peroxireduxin oxidation, cytochrome c release, and caspase activation. Oxidation of peroxireduxin (hydrogen peroxide-reducing enzyme) is considered, to be one of the stress markers indicating the oxidative consequences induced by oxidative stress. Such peroxireduxin oxidation was found to occur 15 min after exposure to 50 μM hydrogen peroxide (Saito et. al. 2007). The secondary products, generated thereby, including p-quinone, participate in caspase-3 activation in a cytochrome *c*-independent pathway (Saito et. al. 2007). Furthermore, there is evidence to show that extracellular autoxidation, which occurs through the generation of hydrogen peroxide and p-quinone, also plays an important role in 6-OHDA-induced cytotoxicity (Saito et. al. 2007). (Фиг. 25).

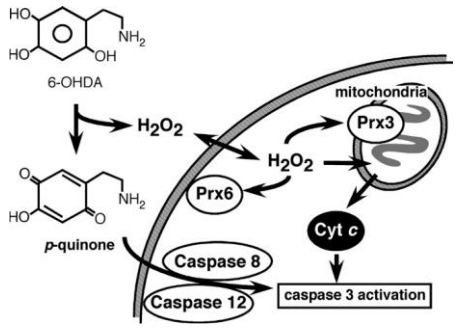

Figure 25. Pathways for 6-OHDA-induced PC12 cell death *(Saito et. al. 2007). 6-OHDA-induced PC12 cell death via H2O2-dependent and -independent mechanism of action. The added 6-OHDA is rapidly oxidized and generates both H_2O_2 and p-quinone. The generated H_2O_2 induces Prx oxidation, cytochrome c release, and caspase-3 activation. On the other hand, the generated p-quinone can induce caspase-3 activation by activating caspase-8 and caspase-12 in a mitochondria-independent manner.*

In view of the above literature data, we performed two series of experiments. In one, 6-OHDA was introduced as the remaining inhibitors in vitro 25 min before obestatin. In order to allow more time for its longer-term cytotoxic effects, we also conducted a series of experiments in which it was injected into the experimental animals one hour before the start of the experiment. Consistent with the above sympathectomy data, our results show that in both series of

experiments as a result of 6-OHDA applications, obestatin did not exert its effect (Figs. 20 and 22). This confirms the assumption that the inotropic effect of obestatin occurs through the nerve endings. The obtained results give us reason to consider that obestatin activates an unknown Gs-coupled receptor in the membrane of the nerve endings and leads to the subsequent activation of the MAPK-pathway (Sazdova et. al. 2009). Activation of the intracellular signaling chain leads to an increase in cAMP and activation of PK A → MEK1/2 → ERK1/2. Administration of PK A (Rp-cAMPS) and MEK1/2 (U0126) inhibitors completely abolished the effect of obestatin (Sazdova et. al. 2009). The activation of both PK A and ERK1/2 phosphorylate the key enzyme for adrenaline synthesis - tyrosine hydroxylase. This leads to the synthesis of new adrenaline molecules and their release in the synaptic terminal. This is confirmed by the lack of effect of obestatin in the presence of the administered tyrosine hydroxylase (3-IT) inhibitors and of key transporters on which epinephrine release depends (reserpine and desipramine). Adrenaline released from the varicosities of the sympathetic endings exerts a powerful control over cardiac activity by increasing the frequency and force of heart contractions. In the isolated heart preparation, obestatin was introduced directly into the ventricle, thereby avoiding the influence on the excitatory conduction system of the heart. This is the reason why only the positive inotropic effect of the administered obestatin was observed.

The positive inotropic effect of obestatin is realized through activation of adrenergic receptors. The interaction of adrenaline with adrenergic receptors shows a temperature dependence both in terms of the receptor with which it will react and in terms of the sensitivity of the receptors to adrenaline (Kunos & Nickerson 1976). At the temperature conditions under which our experiments were conducted (18-22 ºC), adrenaline interacts with equal affinity with both α- and β-adrenoceptors (Kunos & Nickerson 1976). They are G-protein coupled receptors, but pharmacologically they are divided into several subtypes – α_1- and α_2-subtype and β_1-, β_2- and β_3-subtype respectively. Separately, α_1 are a heterogeneous group and are subdivided into three subtypes (Rang et. al. 2003).

All β-adrenoceptor subtypes and α2-adrenoceptors affect the adenylate cyclase (AC)–PC A signaling pathway, but are coupled to different G-proteins that activate or suppress AC activity. β1 - receptors bind to Gs-protein and activate AC, as a result of which the

concentration of cAMP increases and PK A is activated (Rang et. al. 2003, Boron & Boulpaep 2012) α_2 activate Gi/o-protein, which suppresses AC, cAMP concentration decreases and, respectively, PK A activity also decreases. In addition, β_2 can switch from Gs to Gi-proteins after phosphorylation of the receptor by the already activated PK A (Zamah et. al. 2002). PK A phosphorylates a number of target proteins depending on the cell type. In the case of cardiac cells, it phosphorylates L-type calcium channels and KCNQ (Kv7) channels (Hille 2001). As already mentioned in the literature review, the influx of calcium ions from the extracellular environment is essential for the contraction of frog cardiomyocytes and its force is proportional to the influx of calcium (Niedergerke 1963). Previous experiments to verify the involvement of this signaling pathway in the realization of the effect of obestatin showed that inhibition of β-adrenoceptors and PK A completely abolished the inotropic effect of obestatin (Sazdova et. al. 2009).

Studies have shown that in addition to initiating the intracellular chain of events, activation of the G-protein coupled receptor involves two other events. One is phosphorylation of the activated receptor to reduce second messenger generation and the second is internalization of the phosphorylated β-adrenoceptor and resensitization by dephosphorylation, which occurs by protein phosphatase 2A in the early endosome (Vasudevan, at al. 2011) Once the extracellular signal has elicited a cellular response, the initiated signal within the cell must be terminated. This is achieved either by the endocytosis of the receptor or by its desensitization by phosphorylation of the β-adrenoceptor. This results in binding to β-arrestin, which physically prevents G protein binding and blocks the signaling cascade downstream of the receptor. This set of complex events and the precise balance in this process indicates that receptor function is a finely tuned process. Dysregulation of any of these events would alter receptor function and intracellular signaling events (Vasudevan et. al. 2011).

Activated G protein-coupled receptors are generally thought to rapidly desensitize over a period of minutes by phosphorylation and internalization after repeated or prolonged stimulation. This transient activation of G protein-coupled receptors, however, is inconsistent with many observed long-term cellular and physiological responses *in vitro* and *in vivo*. In primary cardiomyocytes and neurons, catecholamine-activated β1-adrenoceptor was found to

continuously stimulate adenylate cyclase for cAMP synthesis, which lasted for more than 8 hours (Fu et. al. 2014). Thus, activated cardiac β-adrenoceptors via the sympathetic nervous system provide a sustained contractile response of the heart during long periods of physical activity, and stimulation of β-adrenoceptors in neurons promotes long-term potentiation, which is critical for learning and memory. Consistent with these functional results, β1-adrenoceptors undergo minimal internalization in primary cardiomyocytes after agonist stimulation due to its low affinity binding to arrestin and its attachment to skeletal proteins such as SAP97 in the sarcolemma (Hu et. al. 2000). The possibility of prolonged stimulation of β-adrenoceptors is also supported by our results, where exogenously added adrenaline exerted a stronger effect in experiments in which the effect of obestatin was not blocked compared to those in which obestatin-induced adrenaline release was absent after treatment with the respective inhibitors (Table 3).

Evidence suggests that ROS play an important role in mediating adrenergic function. Increased ROS production following β-adrenoceptor activation by NADPH oxidase not only contributes to altering downstream signals but also to the feedback loop that modulates receptor phosphorylation and internalization (Xu et. al. 2011). This specific feedback loop of ROS is possible due to their capacity for oxidative and nitrosylation modifications of a number of regulatory proteins, potentially including receptors (Donoso et. al. 2011). Such modification in components of the β-adrenoceptor desensitizing pathway is known to contribute to altered receptor function (Ozawa et. al. 2008). Nitrosylation of G-protein coupled receptor kinases suppresses receptor phosphorylation and reduces desensitization (Vasudevan et. al. 2011). Furthermore, exposure of vascular smooth muscle cells to exogenous hydrogen peroxide causes a rapid increase in cytosolic free calcium concentration, induced by the activation of phospholipase Cλ (FLSCλ) via a tyrosine kinase-mediated pathway (González-Pacheco et. al. 2002). FLSλ (FL C isoform activated by tyrosine phosphorylation) is able to trigger calcium release in several cellular systems by catalyzing the production of inositol triphosphate from membrane phospholipids (Gonzalez-Pacheco et. al. 2000). The activation of PL C is part of the signaling pathway of the other adrenoceptor subtype - α1. It is coupled to Gq/11 protein which activates FLSIt is a membrane-bound enzyme and breaks down the phospholipid

phosphotidylinositol 4,5-diphosphate to diacylglycerol (DAG) and inositol triphosphate (ITP). Each of these secondary mediators elicits respective cellular responses. DAG activates PK C, which in turn phosphorylates its respective target proteins. And ITP affects calcium channels on the membrane of the endoplasmic reticulum and induces the release of calcium ions from intracellular stores. All three α1-adrenoceptor subtypes activate this signaling pathway (Graham et al. 1996). This sequence of events may explain the increased force of cardiac contractions in experiments performed in the presence of 6-OHDA alone (Figs. 20 and 22), which is commensurate with the pure effect of the peptide (Fig. 13). In combination with obestatin, however, 6-OHDA lost its effect on muscle tissue and the amplitude of contractions was close to control values (Figs. 21 and 23).

VI. CONCLUSIONS

1. In the presence of 3-IT, the positive inotropic effect of obestatin is reduced.

2. Vesicular transport and reuptake mechanisms are essential for adrenergic signaling in the presence of obestatin. Treatment with reserpine and desipramine completely abolished the positive inotropic effect of obestatin.

3. Chemical sympathectomy with 6-OHDA eliminate the obestatin effect both in vitro and after pretreatment with this toxin.

4. The presence of 6-OHDA affected not only the effect of obestatin on cardiomyocytes and sympathetic adrenergic signaling, but also exerted a nonspecific positive inotropic effect most likely due to generation of reactive oxygen radicals in cardiomyocytes.

5. The positive inotropic effect of obestatin on isolated frog heart preparations occurs mainly by activating the secretion of adrenaline from the axons of sympathetic autonomic neurons located in the heart muscle wall**.**

VII. SCIENTIFIC CONTRIBUTIONS

A pharmacological approach was developed to identify the target tissue of physiological regulation by a catecholamine-dependent mechanism by studying the sequential inhibition of their synthesis, secretion, reuptake and adrenergic innervation on the effects of these mediators.

Publications included in the dissertation

1. **Bilyana Ilieva,** Hristo Gagov, Mariela Chichova, Iliyana Sazdova, Comparison of Catecholamine Physiological Effects in Vertebrates: Systematic Review, Acta Zool. Bulg., 72 (2), June 2020: 171-178
2. **Bilyana Ilieva,** Hristo Gagov, Iliyana Sazdova, Pharmacology of cathecholamine biosynthesis and signalling, Current Topics in Pharmacology, 2020, Vol 24: 1-11
3. **Bilyana Ilieva**, Hristo Gagov, Mariela Chichova, Iliyana Sazdova, Role of autonomic nevous system in the inotropic effect of obestatin, Current Topics in Pharmacology, 2022, Vol 26: 31-37

Participation in conferences and scientific forums on the subject of the dissertation work

1. **Bilyana Ilieva,** Iliyana Sazdova, Hristo Gagov, Physiology and pharmacology of cathecholamines signaling, International Scientific conference "Kliment's days", 8-9. 11. 2018, Sofa, p 115
2. **Bilyana Ilieva**, Iliyana Sazdova, Hristo Gagov, Effect of obstatin on contractility of excised frog heart after chemical sympathectomy with 6-hydroxydopamine, International Scientific conference "Kliment's days", 8-9. 11. 2018, Sofa, p 114
3. **Bilyana Ilieva,** Iliyana Sazdova, Hristo Gagov, Effect of obestatin on contractility of excised frog heart preparations after treatment with reserpine, Youth scientific conference "Kliment's days, 8.11.2019, Sofia, p 56
4. **Bilyana Ilieva**, Iliyana Sazdova, Hristo Gagov, Effect of obestatin on contractility of excised frog heart preparations after treatment with desipramine, Scientific conference "Kliment's days, 5.11. 2020, Sofa, p 88
5. **Bilyana Ilieva**, Iliyana Sazdova, Hristo Gagov, „Effect of obestatin on maximal force of contraction of excised frog heart after chemical sympathectomy with 3-iodothyrosine", Scientific conference "100 years since of birth of professor

Leon Mitrani, Bulgarian Academy of Sciences, 16. 12. 2021, Sofia