UNIVERSITÀ DEL PIEMONTE ORIENTALE

School of Medicine

***Master of Science in Medical Biotechnologies***

Department of Health Science

EXPERIMENTAL THESIS

# *CHROMOGRANINS AND THE CARDIOVASCULAR SYSTEM*


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# *SUMMARY*

# ABSTRACT

Chromogranins are a family of glycoproteins expressed in neuroendocrine tissues and co-released with cathecolamines and peptide hormones through regulated exocytosis. Chromogranins act both as intracellular regulators of secretory granule biogenesis and as extracellular precursors of bioactive peptides, such as catestatin, vasostatins and secretoneurin, which exert autocrine, paracrine and endocrine effects on the cardiovascular system.

In this study there is an investigation about the role of chromogranins, with focus on Chromogranin B in cardiovascular homeostasis and endothelial cell function. The study is focused on the effects of chromogranins on cell viability, nitric oxide (NO) release, mithocondroial membrane potential, reactive oxygen species production, gluthatione (GSH) levels and intracellular calcium concentration in human vascular endothelial cells (HUVECs). Subsequently, HUVECs were treated with Chromogranin B in the presence or absence of β-adrenergic receptor agonists and antagonists, as well as specific intracellular signaling pathway inhibitors, under physiological conditions or oxidative stress induced by hydrogen peroxide.

In the results, Chromogranin B appears to exert protective effects against oxidative damage and mitochondrial dysfunction, suggesting a potential role in preventing endothelial impairment and cardiovascular disease progression. Chromogranin B may therefore represent a promising target for future therapeutic strategies aimed at preserving endothelial integrity in cardiovascular pathologies.

# INTRODUCTION

## 1.1 Chromogranins: Characteristics and Biological Role

### 1.1.1 Structure and function of chromogranins

The Chromogranins belong to the family of water-soluble acid glycoproteins stored in the matrix of dense secretion granules, containing some molecules and hormones of endocrine and neuroendocrine systems. So, they are released such as peptide hormones, neurotransmitters or amines in response to a variety of physiological stimuli.

Granins made of single-polypeptide chains of 180 to 700 amino acid residues, containing as amino-terminal signal peptide that directs the movement of the preproteins from ribosomes to the endoplasmic reticular lumen and the Golgi complex in which post-translational modification occur. Granins tend to bind to calcium and these characteristics mean that granins have functions within the core of secretory granules.

Proteins are secreted from cells by exocytosis: these granules release their contents only in response to a stimulus that is specific for a particular type of cell (Feldman, 2003).

The chromogranin family includes the following members:

| **Chromogranins** | **Synonym** |
|---|---|
| Chromogranin A (CgA) | parathyroid secretory protein 1 |
| Chromogranin B (CgB) | secretoneurin, secretogranin I (SgI) |
| Chromogranin C (CgC) | secretogranin II (SgII), secretoneurin-included (SN, included) |
| Secretogranin III/1B1075 (SgIII) | |
| Secretogranin IV/HISL-19 antigen (SgIV) | |
| Secretogranin V | neuroendocrine protein 7B2 |
| Secretogranin VI | NESP55 |

**Figure 1**: The image presents a table listing chromogranins and secretogranins along with their respective synonyms. Chromogranins and secretogranins are proteins associated with the secretory vesicles of neuroendocrine cells (Louthan, 2011).

These secretoproteins do not share too many structural similarities. Just one short region, located in the C-terminal motif, is present in all them. Chromogranin A and B have a similar region in their N-terminal sections and it's composed by two cysteine residues involved in a disulphide bound. This structure does not occur in Secretogranin II (Louthan, 2011).
Chromogranin family are present in a lot of quantity in the neuroendocrine tissue, so it means that are involved in a multiple function that can divided in intracellular and extracellular: for the first, an environment characterized by low pH and high concentration of calcium induced the aggregations of the granins (Louthan, 2011).
They can interact with other components of the matrix of the secretory granule, such as catecholamines, serotonin and histamine. Then these molecules are able to module of peptide hormone and neuropeptide processing.
Outside of the cell, the presence of paired amino acids in granins suggest that they function as prohormones giving rise to bioactive peptides as a result of post-translational proteolytic processing. Some of these, such as Chromogranin A, Chromogranin B and Secretogranin II have autocrine, paracrine and endocrine activities (KB., 2010).

### 1.1.2 Genomic organization and transcriptional regulation

The chromosomal positions of all granins, except secretogranin IV, have been identified in humans, cows, mice, and rats. In each species, these genes are located in regions of chromosomes that share a conserved gene order across species. Studies using Northern blot analysis of messenger RNA have shown that granins are distributed throughout neuroendocrine tissues with regulated secretory pathways. Granin production varies among cell types in response to agents like cAMP, steroid hormones, neurotrophins, and phorbol esters (Taupenot, 2003).
Research using granin-gene promoters in transfected cells has revealed the mechanisms that regulate both constitutive and stimulus-induced expression of granins. The promoter regions of chromogranin A, chromogranin B, and secretogranin II share a cAMP-response element (CRE) upstream of the TATA box, though their promoter sequences differ otherwise. This CRE site plays a key role in the expression specificity of these granins in neuroendocrine cells, explaining how their production is upregulated by factors such as nicotinic–cholinergic agonists and the preganglionic neuropeptide PACAP.
Neuronal differentiation of PC12 cells induced by nerve growth factor increases the expression of chromogranin A and B via the CRE site.

Other promoter elements, such as a serum response element in secretogranin II and G- or C-rich regions in chromogranin B, also influence granin expression. Furthermore, glucocorticoid sensitivity in rat chromogranin A is linked to a unique glucocorticoid-response-element variant (Taupenot, 2003).

### 1.1.3 Tissue distribution and localization

Proteins are secreted from cells through two main pathways: constitutive and regulated exocytosis.

The constitutive pathway is found in all cell types and allows to produce newly synthesized proteins that are transported from the Golgi network to the plasma membrane in vesicles for immediate release. The secretion, in this pathway, is directly linked to the rate of protein synthesis. Instead, regulated secretory pathway is present in specialized cells like neuroendocrine cells and neurons and some proteins (such as hormones and neurotransmitters) are stored in secretory granules that release their contents only in response to specific stimuli (Taupenot, 2003).

After the release, the sorting of granins into the regulated pathway occurs at the trans-Golgi network. In this process several factors are involved, for example there is a selective aggregation of proteins under conditions of high calcium and mildly acidic pH.

Granins are widely distributed across the endocrine, neuroendocrine, central and peripheral nervous systems. CgA is in neurons of the cerebellum, cerebral cortex, septum, and amygdala and possibly in astroglial cells. Chromogranin B and Secretogranin II share a similar distribution but vary in quantity depending on the type of neuroendocrine cell. Secretogranin V is present throughout the central nervous system and in various neuroendocrine tissues. Secretogranin VI is located in the central nervous system, adrenal medulla, anterior and posterior pituitary and intestine (Taupenot, 2003).

### 1.1.4 Intracellular and extracellular functions of granin family

The formation of secretory granules involves several granins, such as Chromogranin A, which aggregate under conditions of low pH and high calcium levels. These granins interact with other components of the secretory granule matrix, such as catecholamines, serotonin, and histamine, indicating that they play a key role in granule formation.

Granins are crucial in the formation of secretory vesicles, facilitating the assembly of storage complexes and acting as chaperones to properly sort regulated secretory proteins. Specifically, Chromogranin A is essential for the formation of granules and the storage of hormones in neuroendocrine cells. Its absence leads to a depletion of secretory granules, inhibits the regulated secretion of prohormones, and reduces granule-associated proteins. However, the regulated secretory phenotype can be restored by reintroducing Chromogranin A into deficient cells via transfection (Taupenot, 2003).

Granins are proproteins that contain multiple recognition sites for endopeptidases, such as prohormone convertase 1 and 2 (PC1 and PC2) and plasmin. These enzymes, particularly the serine endoproteases PC1 and PC2, play a key role in processing granins into active peptides.

Secretogranin V (7B2) is unique because a peptide from its carboxy-terminal selectively inhibits PC2, while its amino-terminal acts as a chaperone for PC2 in the endoplasmic reticulum, aiding in the activation of pro-PC2. In mice lacking 7B2, the absence of PC2 activity leads to impaired processing of pancreatic islet hormones, resulting in hypoglycemia, elevated levels of proinsulin (hyperproinsulinemia), and reduced glucagon levels (hypoglucagonemia). This highlights the critical role of 7B2 in regulating PC2 activity and maintaining proper hormone balance.

For extracellular functions, the granin family is involved in the production of bioactive peptides that have a role in autocrine, paracrine and endocrine regulation (Taupenot, 2003). Their post-translational processing gives rise to peptides with diverse biological activities, such as pancreastatin, vasostatins, and catestatin that are produced from CgA.

For example, pancreastatin is involved in the inhibition glucose-stimulated insulin release in pancreatic beta cells; besides it cause a reduction of glucose uptake in skeletal muscles, activates glycogen breakdown in the liver and inhibits insulin signaling in adipocytes. It suppresses amylase release from the pancreas, gastric acid secretion, and parathyroid hormone release.

Vasostatin I and II are involved in inhibition of the vasoconstriction in blood vessels and modulate cell adhesion and it suppress parathyroid hormone secretion.

Catestatin blocks catecholamine release by inhibiting the nicotinic cholinergic receptor in chromaffin cells, serving as a negative feedback mechanism; it prevents desensitization of catecholamine release under repeated stimulation and low catestatin levels are associated with hypertension, suggesting its role in regulating blood pressure by modulating adrenal epinephrine release (Taupenot, 2003).

### 1.1.5 Physiological and pathological roles

Numerous studies have shown that granins are present in various endocrine, neuroendocrine, and neuronal tumors and are released into the bloodstream. Their presence in tumors typically reflects their expression patterns in the corresponding healthy tissues (Taupenot *et al*, 2003).

Granins, particularly chromogranin A, are widely used as diagnostic and biological markers for neuroendocrine tumors. CgA is standardly used in immunohistochemical analyses for neuroendocrine tumors, as elevated serum CgA levels indicate the presence of these tumors and can be used to monitor their progression or response to treatment: in children with suspected neuroblastoma, CgA has a sensitivity of 91% and specificity of 100%, making it a reliable diagnostic tool and elevated CgA levels can also occur in conditions like parathyroid, thyroid, or gastric hyperplasia, which limits its ability to distinguish between hyperplasia, adenoma, or carcinoma.

Even CgB and secretogranins II, III, IV, and V are also detectable in various neuroendocrine tumors. Circulating granins are important biological markers for pheochromocytoma, a tumor of the adrenal gland: elevated plasma levels of CgA can be diagnostic for pheochromocytoma, von Hippel-Lindau disease, multiple endocrine neoplasia type II and neurofibromatosis. Even CgB and secretogranin II levels may also aid in diagnosing pheochromocytoma.

In patients with metastatic carcinoid tumors, serum CgA levels can be elevated up to 1000 times above the normal range. The stable nature of CgA makes it a reliable marker for detecting carcinoid tumors and monitoring their progression.

Other diagnostic tests for carcinoid tumors include measuring urinary 5-hydroxyindoleacetic acid, serum serotonin and serum neuron-specific enolase.

In patients with small-cell lung cancers showing neuroendocrine differentiation, plasma chromogranin A levels can serve as a marker for treatment response and be useful for monitoring recurrent disease. However, its low sensitivity makes CgA unreliable as a diagnostic marker for these cancers, even though it can indicate the degree of neuroendocrine differentiation.

In men with prostate cancer, measuring CgA levels can assist in establishing the diagnosis and determining prognosis. Circulating CgA levels may be elevated even if the prostate-specific antigen (PSA) level is normal.

Elevated CgA levels, possibly indicating neuroendocrine differentiation, may also predict a poor response to hormone therapy and suggest a worse prognosis.

CgA immunoreactivity has been found in cells of colorectal tumors and breast tumors, but its diagnostic or prognostic value in these contexts has not been confirmed. Tumors with suspected or partial neuroendocrine differentiation, such as choriocarcinoma, thymoma, malignant melanoma, and renal-cell carcinoma, are not associated with elevated plasma CgA levels.

Increased sympathoadrenal activity may play a role in the development of essential hypertension (which can be idiopathic, familial, or genetic). The release of CgA alongside catecholamines suggests that exocytosis is the primary mechanism for catecholamine release in humans. Basal plasma CgA levels are correlated with sympathetic tone and have a high heritability, as shown in twin studies. Compared to age-matched normotensive controls, individuals with essential hypertension have elevated plasma CgA levels, as well as an increased release of stored CgA in response to insulin-induced hypoglycemia. The pancreastatin fragment of CgA, which is dysglycemic, is also increased in patients with essential hypertension and may contribute to the insulin resistance often seen in these individuals. Another chromogranin A-derived peptide, catestatin, which inhibits catecholamine release, is found to be decreased in patients with essential hypertension and even in normotensive individuals with a family history of hypertension.

This reduction in catestatin is associated with increased epinephrine secretion and enhanced adrenergic responses to stress, indicating that low catestatin levels may increase the risk of developing hypertension (Taupenot, 2003).

### 1.1.6 Chromogranin A (CgA)

CgA is the major member of the chromogranin family of neuroendocrine secretory proteins. Its molecular weight is 48 kD and isoelectric point is 4.9. It is undergone to post translational modifications that include carboxymethylation, glycosylation, phosphorylation and sulphation. CgA has been first identified in chromaffin cells when adrenal gland secretion was analyzed after splanchnic nerve stimulation. It is released by endocrine and neuroendocrine cells, where it's co-expressed together with other polypeptide hormones and neurotransmitters (Louthan, 2011).

The human gene of CgA is a single-copy gene and it's 12,194 base pair in the locus 14q32.12 made by 8 exons giving raise to 2043 nucleotide transcripts.

Many signals are co-expressed with CgA, thus CgA responds to the wide scale of modulation signals influencing CgA expression in various tissues. Steroid hormones influence the CgA gene expression: they are linked to their nuclear and cytosolic receptors and this complex may associate with cis-acting DNA response elements to exert either positive or negative transcriptional control. The distribution of CgA is condensed in granules of adrenal medulla and in the dense-core vesicles of sympathetic nerves. The content may be variable in base of the type of the tissue: central and peripheral nervous system, pituitary gland and parathyroid glands are rich in CgA (Louthan, 2011). (Laguerre, 2019)

It's also present in calcitonin-producing cells of thyroid gland, in exocrine tissue of the pancreas and in insulin and glucagon-producing cells or in placenta. The secretion of CgA is detectable in neuroendocrine system into gastrointestinal tract, spleen, thymus, prostate and lungs.

From neuroendocrine system may be originate a lot of tumors and the present of CgA is signal of the proliferation of the tumor.

During the biosynthesis of CgA, several intracellular messenger are involved, for example protein kinase A, intracellular calcium level and protein kinase C. Then the granins goes into rough endoplasmic reticulum and Golgi apparatus. At the end, when there is a stimulation from neuroendocrine system, the vesicle with granins are released.

CgA is high-capacity and low affinity calcium-binding protein and it's influenced by pH and may change during the process of maturation of secretory granules (Fig. 2).

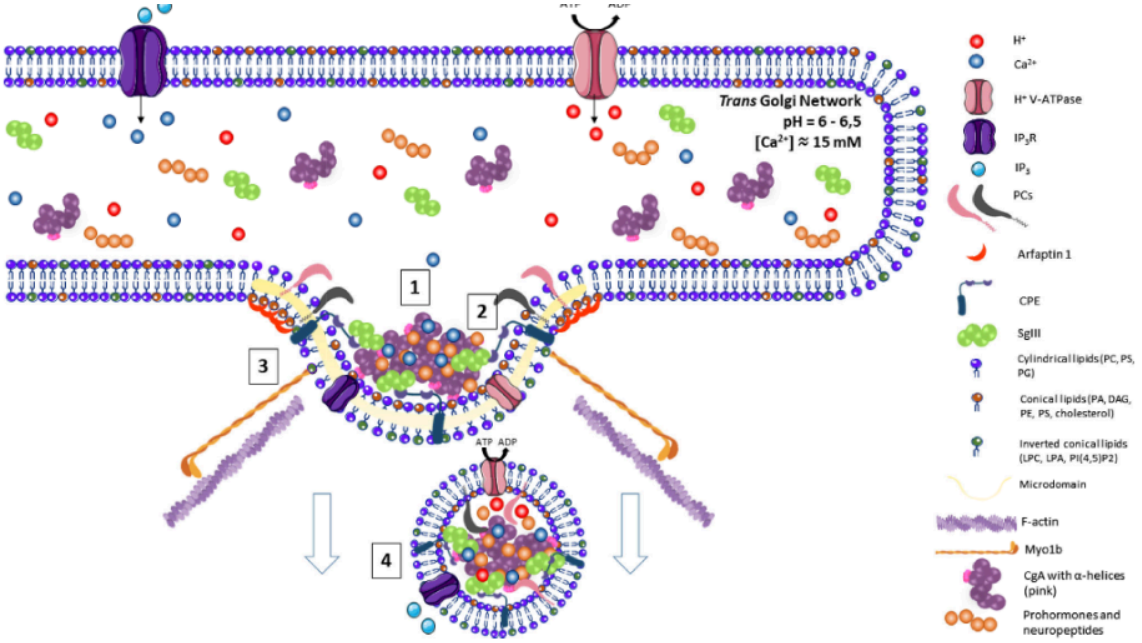



**Figure 2**: This image represents a crucial cellular mechanism for the secretion of neuroendocrine peptides and hormones, which are stored in dense-core secretory granules. The role of chromogranins and secretogranins in this process highlights their importance in regulated exocytosis and hormone secretion (Laguerre, 2019).

They are responsible of the change conformation of the protein and may enhance association of this protein with the membrane. CgA is involved also in granulogenesis (formation of immature structures via the trans-Golgi and the development of secretory vesicles containing nucletotides, neurotransmitters and cations), there is a co-storage and co-release of these components alongside other resident hormones, with regulation of packaging and processing of hormone molecules. Secretory vesicles are stabilized by reducing the effective osmotic pressure within intact chromaffin vesicles, which helps prevent their rupture. Moreover, chromogranins inhibit catecholamine secretion by adrenal medulla, cholecystokinin-induced amylase secretion by exocrine pancreas, pro-opiomelanocortin secretion in neuroendocrine tissues and acid secretion by parietal cells of stomach (Louthan, 2011).

During sympathoadrenal activation, CgA is co-released with catecholamines through exocytosis into the adrenal medulla and sympathetic nerve endings.

CgA levels correlate with norepinephrine during sympathetic stimulation and with epinephrine during adrenal medulla stimulation; however, this correlation is weak at rest and influenced by various tissue factors (Louthan, 2011).

CgA levels rise during intense stimulation, such as strenuous exercise or stress, but rarely exceed twice the normal range, unlike neuroendocrine tumors (NETs), where pathological levels are significantly higher (e.g., in small intestine carcinoids). Elevated CgA levels are also seen in pheochromocytoma, a NET of the adrenal medulla associated with secondary hypertension, though some NET subtypes may only slightly increase CgA levels.
CgA is a particular tumor marker used both in immunohistochemical examination of biopsied tumor tissue and as a serum tumor marker. It's important for the gastroenteropanceratic tumor NET, in pheochromocytoma and in the other endocrine and non-endocrine tumors (Louthan, 2011).

### 1.1.7 Chromogranin B (CgB)

The CgB gene is located in human chromosome 20pter-p12 comprises five exons. This gene shares several features with the CgA gene, including widespread expression in the endocrine system, an acid protein blackbone, a random-coil structure and heat stability.
There are two specific regions, the Cys loop at the N-terminus and a C-terminal region that are significant sequence homology and this conservation is located in non-mammalian vertebrates. After synthesis, CgB undergoes post-translational O-glycosylation and is sorted into secretory vesicles.
CgB is predominantly expressed in neurons and peptidergic endocrine cells. Its presence is prominent in adrenal chromaffin cells and nervous cells (here it's involved in neurotransmitter regulation and synaptic vesicle dynamics) (Bartolomucci *et al*, 2011).
Chromogranin B is also called secretogranin I and it has a several biological functions. For first key role, it's important in the granule biogenesis and hormone storage, contributing to the formation and maintenance of secretory granules. Then it's involved in proteolytic processing, CgB undergoes proteolytic cleavage at specific sites, like dibasic LysArg and monobasic Arg, generating several bioactive peptides. Secretolytin is important for its antimicrobial properties, so it can be involved in innate immunity. Recently high levels of this molecule are been discovered in the patients undergoing cardiopulmonary bypass, where it's released from monocytes and reported to possess antibacterial activity.
Other peptides that derive from CgB are BAM-1745 and PE11, there are important in the regulatory processes (Troger, 2017).
At the end, CgB is involved in the regulation of neuroendocrine secretions, it's possible thanks to its presence in synaptic and endocrine secretory vesicles.

CgB play an important role in diagnostic and therapeutic applications. As a matter of fact it may be a biomarker for neuroendocrine tumor (NETs), because elevated levels of CgB (like high level of CgA) means the presence of NETs. Its presence in the neurons may be a marker of neurodegenerative diseases. CgB has a potential protective role against oxidative stress in endothelial cells. It appears to help maintain mitochondrial function, reduce reactive oxygen species (ROS) production, and preserve cellular viability, which are all essential for preventing endothelial dysfunction (Grossini E, 2024).

Furthermore, CgB interacts with key intracellular signaling pathways such as Pi3K/Akt, MEK1/2/ERK1/2, AMPK, CaMKII, and PKA, which are involved in cell survival, calcium regulation, and endothelial function.

Overall, CgB is a multifunctional protein with significant roles in neuroendocrine secretion, cardiovascular regulation, and cellular protection against oxidative stress. Further studies are needed to fully understand its therapeutic potential, particularly in the context of cardiovascular diseases (Grossini E, 2024).

### 1.1.8 Chromogranin C (CgC)

Chromogranin C, or Secretogranin II, is another member of the chromogranin family of acid glycoproteins. It is located on human chromosome 2q35-2q36, comprises two exons. The first encodes 215 nucleotides of the 5'UTR and the second encodes 14 nucleotides of the 5'UTR plus entire coding region and 3'UTR of SgII. It's a 617-aminnoacid preproprotein with nine pairs of basic amino acids. Proteolytic cleavage at these sites produces intermediate-sized proteins along with many small peptides, including secretoneurin (SN), EM66 and manserin CgC is expressed in endocrine and neuroendocrine cells and, like CgA and CgB is stored in the dense-core secretory granules and it's involved in regulated secretion (Bartolomucci, 2011).

CgC contributes to its role in granule biogenesis and hormone storage. It's majorly distributed in the peripheral nervous systems and into various endocrine glands. It has an important role in the stress responses and in the signaling regulation.

After synthesis, CgC undergoes proteolytic cleavage and this allows to generate several bioactive peptides, for example secretoneurin (SN) (Plášek, 2022).

This molecule is a 33 amino-acid evolutionary conserved neuropeptide and its main effects may be cardioprotective and can mediate through its inhibition of calmodulin-dependent kinase II, that is involved in intracellular calcium handling.

The result is a reduction of the risk of ventricular arrhythmias and heart failure. SN is also involved in the reactive oxygen species concentration, modulating the immune response. Accordingly, this molecule is an important marker of cardiac injury and overload. It can predict the risk of hospital mortality in critically injury ill patients.

SN is involved in several processes, including apoptosis, the immune response, inflammation, calcium handling, arrhythmogenesis and cell cycle regulation (Plášek, 2022). It's a potentially range of clinical utility in cardiology as a biomarker of heart failure or risk of arrhythmogenesis. Therefore it's a marker of neuroendocrine cell tumor activity and as a marker of inflammation (Plášek, 2022).

### 1.1.9 Secretogranin III

The SgIII gene (SCG3), located on human chromosome 15q21, consists of 12 exons. The 3366-bp SgIII mRNA encodes a 468-amino-acid acidic secretory protein containing seven consecutive pairs of basic amino acids, which is highly conserved across evolution, from mammals to fish. SgIII is produced as an N-glycosylated protein and undergoes proteolytic cleavage within secretory vesicles, resulting in intermediate-sized proteins. However, no biologically active peptides derived from SgIII have been identified to date (Bartolomucci, 2011).

In the nervous system, SgIII is involved in neuronal communication and neurotransmitter release.

It has been implicated in apoptosis signal transduction as a caspase substrate, suggesting a role in programmed cell death. Additionally, dysregulated expression of SgIII has been associated with neurotoxin-induced dopaminergic neuron apoptosis, indicating its potential involvement in neurodegenerative processes (Zhi-Hao Huang, 2012).

SgIII is also expressed in astrocytes, a type of glial cell in the central nervous system.

Its expression is finely regulated during glial activation, implicating SgIII in the astrocyte secretory pathway in vivo. Overexpression of SgIII has been observed in reactive astrocytes, suggesting a role in the response to neural injury or disease.

This is a multifunctional protein involved in the formation of secretory granules, neuronal communication, and potentially in disease processes such as neurodegeneration, reactive gliosis, pathological angiogenesis, and metabolic disorders. Ongoing research continues to elucidate its diverse roles and therapeutic potential (Sonia Paco, 2010).

### 1.1.10 Secretogranin V

Human 7B2 (SCG5), located on chromosome 15q13-q14, consists of six exons and produces two mRNA variants of 1244 and 1241 base pairs. The longer variant encodes a protein that is one amino acid longer than the shorter one due to an alternative in-frame splice junction. Among the granin proteins, 7B2 and proSAAS have the least acidic isoelectric points (pI) and share structural and functional similarities. Both contain a C-terminal peptide that inhibits the catalytic activity of prohormone convertases (PCs).

7B2 is considered one of the most evolutionarily conserved granins, particularly in vertebrates, but also has orthologs in invertebrates like *Aplysia*, *Caenorhabditis elegans* and *Drosophila*.

Two regions of 7B2 are highly conserved: a proline-rich sequence essential for its role as a chaperone for PC2, and a C-terminal peptide that inhibits PC2's catalytic activity.

Comparisons between the expression patterns of PC2 and 7B2 (where PC2 is expressed in a subset of 7B2's locations) and studies of knockout mice highlight 7B2's broader role (Bartolomucci, 2011).

Mice lacking 7B2 develop a lethal form of Cushing's disease, while PC2-null mice remain largely healthy, suggesting that 7B2 may act as a chaperone for other proteins in addition to PC2.

Additionally, Secretogranin V has been identified as a potential diagnostic biomarker for pancreatic acinar carcinoma (PAC). Studies suggest that circulating SCG5 levels may be associated with adipopinia, making it a promising biomarker for PAC diagnosis.

Secretogranin V is a multifunctional protein involved in the proper folding and transport of other secreted proteins, with significant implications in various pathological conditions, including neurodegenerative and metabolic disorders, as well as certain types of cancer (Yunju Jo, 2022).

### 1.1.11 Secretogranin VI or NESP55

NESP55 is part of the highly intricate imprinted GNAS gene locus on human chromosome 20q13.2, which is responsible for encoding the α-subunit of the stimulatory G protein (Gsα). This locus utilizes multiple promoters and distinct first exons to produce mRNA for several unique proteins, including Gsα, NESP55, XLas, 1A, and antisense transcripts (referred to as nespas in mice).

The NESP55 exon, which contains the 5'-untranslated region along with the full open reading frame of NESP55, is spliced onto exons 2–13 of Gsα.

This specific splicing pattern has been observed in all species studied so far. Furthermore, additional splicing in the 3'-untranslated region results in a prominent shorter mRNA variant. NESP mRNA can undergo genomic imprinting, meaning they are transcribed exclusively from the maternal allele. The NESP55 protein, composed of 244 amino acids, contains six pairs of consecutive basic amino acids in its sequence. These sites are cleaved to generate smaller peptides, including the C-terminal peptide GAIPIRRH (Bartolomucci, 2011).

## 1.2 The Cardiovascular system: basic anatomy and physiology

### 1.2.1 Anatomy of the heart and blood vessels

#### *1.2.1.1 Heart anatomy*

The heart is a muscular organ located slightly to the left of the chest cavity. It functions as a pump, circulating blood through two main circuits: the pulmonary circuit and the systemic circuit. It is a hollow, muscular, cone-shaped organ located in the middle of the mediastinum, enclosed by the pericardium.

It lies behind the body of the sternum, with about one-third on the right side and two-thirds on the left of the midline. The heart measures approximately 12 x 8.5 x 6 cm and weighs around 310 g in males and 255 g in females (Fiocca, 2000).

Its primary function is to pump blood throughout the body to supply tissues with the necessary nutrients. The term "cardia," derived from Greek, refers to the heart and gives rise to the adjective "cardiac."

The heart is divided into four chambers: upper left and right atria and lower left and right ventricles. In a healthy heart, blood flows one way through the heart due to heart valves which prevent backflow (Fig.3). The wall of the heart is made up of three layers that are epicardium, myocardium and endocardium (Fiocca, 2000).

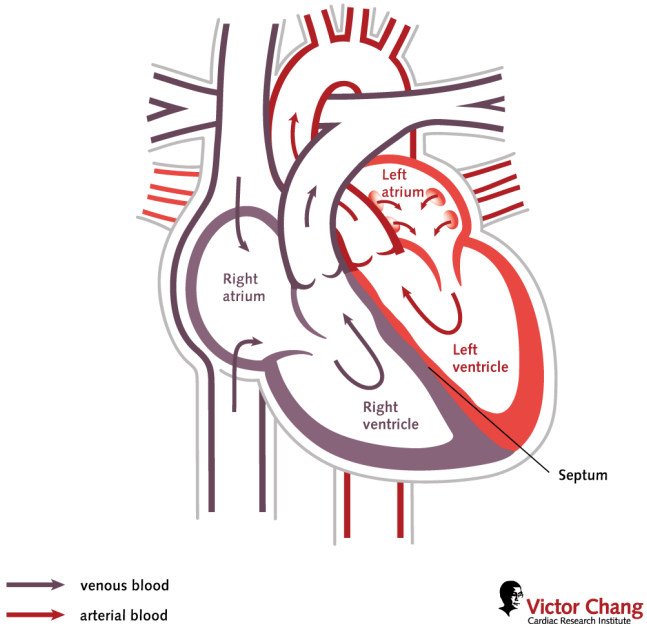



**Figure 3**: In this picture it's illustrated the blood circulation inside the heart (Chang).

In the heart there are some specific cells that are involved in the pumping of the blood flow: pacemaker cells. These generate an electric current that causes the heart to contract, traveling through the atrioventricular node and along the conduction system of the heart.

Deoxygenated blood enters the heart through the right atrium from the superior and inferior venae cavae and passes to the right ventricle. From here, it is pumped into pulmonary circulation to the lungs, here it receives oxygen and give off carbon dioxide.

Oxygenated blood then returns to the left atrium, passes through the left ventricle and is pumped out through the aorta into systemic circulation, traveling through arteries, arterioles and capillaries, before being returned to the heart through venules and veins (Hall, 2020).

The heart contains four valves that separate its chambers. The atrioventricular valves are located between the atria and ventricles. The tricuspid valve is found between the right atrium and right ventricle, while the mitral (or bicuspid) valve is positioned between the left atrium and left ventricle. Papillary muscles extend from the heart walls to the valves via fibrous cords called chordae tendinae, which prevent the valves from moving too far backward when they close. In addition, there are two semilunar valves located at the exits of each ventricle.

The pulmonary valve is at the base of the pulmonary artery, where it prevents blood from flowing back into the ventricle when it relaxes. The aortic semilunar valve, located at the base of the aorta, also functions similarly and is not attached to papillary muscles.

The heart is made up of three layers (Fig.4): endocardium (the innermost layer lining the heart chambers), myocardium (the muscular middle layer responsible for contracting and pumping blood) and epicardium (the outer layer, also known as the visceral pericardium, which forms part of the pericardium) (Testut, 1998).

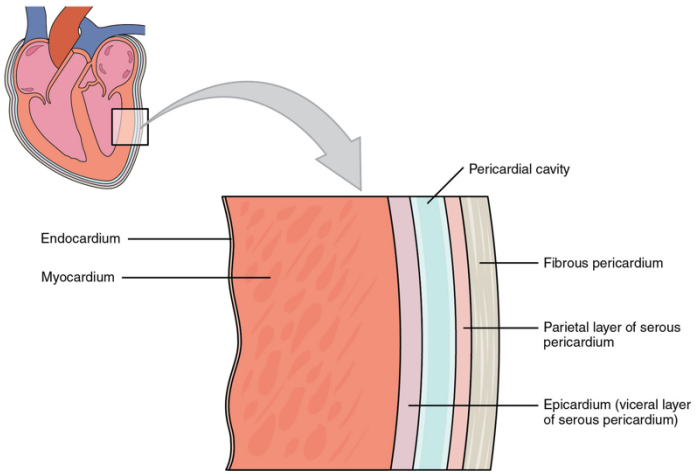



**Figure 4**: In this figure the layers of the heart are illustrated: endocardium, myocardium and pericardium (Chang).

### *1.2.1.2 Vascular system*

The circulatory system is made up of two main circuits: pulmonary circulation and systemic circulation (Fig.5).

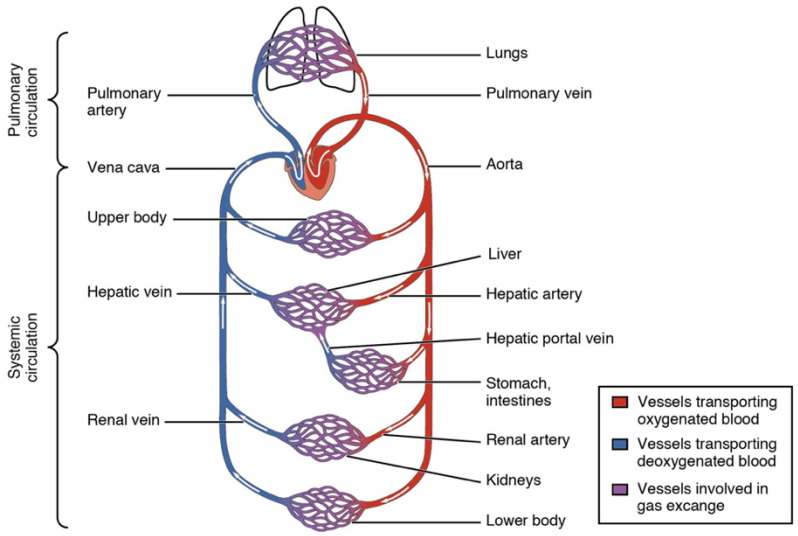



**Figure 5**: In this picture, the vascular system, composed by two different circulations, the pulmonary circulation and systemic circulation, is shown (OpenStax, 2013).

Pulmonary circulation starts from the right side of the heart, transporting deoxygenated blood to the lungs where it is oxygenated and then returned to the left side of the heart. The systemic circulation carries oxygenated blood from the left heart to the body and brings deoxygenated blood back to the right heart through the vena cava. The systemic circulation can be further divided into microcirculation and macrocirculation.

Additionally, there are specialized circulatory routes, including coronary circulation for the heart, cerebral circulation for the brain, renal circulation for the kidneys, and bronchial circulation for the lungs. The human circulatory system is a closed system, where nutrients are delivered to organs through tiny blood vessels in the microcirculation. A key role of the system is also working with the immune system to defend against infections (Jain, 2023).

The blood vessels in the circulatory system include arteries, veins, and capillaries. Oxygen-rich blood enters the systemic circulation from the left ventricle through the aortic semilunar valve. The aorta arches and gives off branches that supply the upper body before passing through the diaphragm at the level of the tenth thoracic vertebra, entering the abdomen. It continues downward, providing branches to the abdomen, pelvis, perineum, and lower limbs (Anderson, 2004).

The aorta's walls are elastic, which helps maintain blood pressure throughout the body.
As the aorta receives about five liters of blood from the heart, it recoils, contributing to the pulse of blood pressure. As the aorta branches into smaller arteries, their elasticity decreases while their compliance increases. Arteries branch into smaller arterioles, which then lead to capillaries.
Capillaries converge into venules, which further combine into veins.
The venous system drains into two major veins: the superior vena cava, which primarily collects blood from tissues above the heart, and the inferior vena cava, which drains tissues below the heart. These two large veins empty into the right atrium of the heart. Generally, arteries branch into capillaries, which then converge into veins that return blood to the heart. An exception to this rule is the portal veins, such as the hepatic portal vein, which collects blood from capillaries around the gastrointestinal tract. This blood, rich in the products of digestion, does not return directly to the heart but instead passes through a second capillary system in the liver (Kienle, 1996).
The heart receives oxygen and nutrients through a small "loop" within the systemic circulation, rather than from the blood in its four chambers. The coronary circulation system is responsible for supplying blood to the heart muscle. It starts near the beginning of the aorta with two coronary arteries: the right coronary artery and the left coronary artery.
After providing nourishment to the heart muscle, blood returns through the coronary veins into the coronary sinus, which then empties into the right atrium. The backflow of blood during atrial contraction is prevented by the Thebesian valve. The smallest cardiac veins drain directly into the heart chambers (Anderson, 2004).

## 1.2.2 Neuroendocrine regulation of the cardiovascular system

### *1.2.2.1 Autonomic nervous system (ANS) control*

The autonomic nervous system (ANS) is part of the peripheral nervous system that controls involuntary body functions, such as heart rate, blood pressure, breathing, body temperature, sweating, and digestion, ensuring homeostasis. The ANS operates automatically, without conscious control, but is regulated by centers in the spinal cord, brainstem, and hypothalamus.
The ANS consists of two main components: the sympathetic and parasympathetic systems. These systems have opposing effects on the body.

The sympathetic system prepares the body for action in stressful situations ("fight or flight") by releasing norepinephrine (NE), which increases heart rate, blood pressure, and other responses like pupil dilation, sweat secretion, and reduced digestion. In contrast, the parasympathetic system, active during restful states, releases acetylcholine (ACh) to slow the heart rate, constrict pupils, and promote digestion (Gordan, 2015).

Both systems typically work together to balance body functions, although some organs only respond to one system.

For instance, blood vessels are regulated by the sympathetic system, which maintains a constant state of tone, with vasodilation occurring when sympathetic stimulation decreases. During rest, the parasympathetic system predominates, controlling the heart rate at around 60-75 bpm.The ANS pathways consist of a two-neuron chain: a preganglionic neuron and a postganglionic neuron. In the sympathetic system, nerves originate from the thoracolumbar region of the spinal cord (T1-L2), while parasympathetic nerves originate from the brainstem and sacral spinal nerves (S2-S4). Sympathetic nerves have short preganglionic and long postganglionic neurons, while parasympathetic nerves have long preganglionic and short postganglionic neurons, with the vagus nerve carrying most parasympathetic fibers.

The sympathetic nervous system is the part of the autonomic nervous system (ANS) that regulates the body's response to stress or emergency situations, known as the "fight or flight" response, while the parasympathetic nervous system is responsible for basal organ functions (Marieb, 2018).

The cardiac sympathetic preganglionic nerves (typically all myelinated) originate from the upper thoracic segments of the spinal cord (T1-T4).

After traveling a short distance, these fibers leave the spinal nerves through branches called white rami and enter sympathetic ganglia, forming the sympathetic chain ganglia along the sides of the spinal column. The postganglionic neurons extend to organs, such as the heart (Gordan, 2015).

Neurotransmitters are chemical substances that transmit signals between neurons and target cells across a synapse. While preganglionic neurons in both the sympathetic and parasympathetic systems release acetylcholine (ACh), the postganglionic terminals of the sympathetic nervous system release norepinephrine (NE), which resembles epinephrine (adrenaline). These sympathetic postganglionic fibers are therefore referred to as adrenergic fibers. There are two types of adrenergic receptors: β and α and in the cardiovascular system, there are β1, β2, α1, and α2 adrenergic receptors.

Parasympathetic activity generally produces effects opposite to those of sympathetic activation. Unlike the sympathetic nervous system, the parasympathetic system has minimal impact on myocardial contractility.

It has a negative chronotropic effect (decrease in heart rate): the vagus nerve directly innervates the sinoatrial (SA) node, and when activated, it lowers the heart rate, leading to a negative chronotropic effect.

Negative inotropic effect (decrease in myocardial contractility): it is debated whether parasympathetic stimulation reduces myocardial contractility, as the vagus nerve does not directly innervate cardiomyocytes beyond the SA and atrioventricular (AV) nodes. However, recent studies suggest it may affect the atrium (Gordan *et al*, 2015).

Negative dromotropic effect (decrease conduction velocity): parasympathetic stimulation inhibits AV node conduction velocity, reducing the speed of electrical signals in the heart (Gordan, 2015).

#### *1.2.2.2 Hormonal regulation*

Cardiovascular function is influenced not only by the autonomic nervous system (ANS) but also by various endocrine hormones. Epinephrine, dopamine, and norepinephrine, released by the adrenal glands, play a key role in the "fight or flight" response, while hormones such as vasopressin, renin, angiotensin, aldosterone, and atrial natriuretic peptide help regulate blood pressure through water reabsorption (Gordan, 2015).

A notable exception in sympathetic fibers is the preganglionic fibers that pass through sympathetic ganglia to the adrenal medulla. These fibers stimulate chromaffin cells, which release 20% norepinephrine and 80% epinephrine. Epinephrine and norepinephrine activate sympathetic receptors in the cardiovascular system, influencing heart rate and blood pressure. Dopamine, which has limited actions in the autonomic system, can excite or inhibit receptors and is converted to norepinephrine, further increasing heart rate and blood pressure.

Epinephrine, produced from phenylalanine and tyrosine in the adrenal medulla, can stimulate all major adrenergic receptors (α1, α2, β1, and β2). At low concentrations, it is β2-selective, causing vasodilatation; at high concentrations, it also activates α1, α2, and β1 receptors, leading to vasoconstriction and increased heart rate and contractility. Blood pressure is regulated by vasoconstriction and vasodilatation, where changes in vessel diameter significantly impact blood pressure.

Epinephrine is crucial in initiating the fight-or-flight response, enhancing oxygen and glucose delivery to the brain and muscles by increasing cardiac output and promoting vasodilatation (Gordan, 2015).

Vasopressin (antidiuretic hormone) is released during hypovolemic shock to help regulate blood pressure and maintain organ perfusion by promoting water retention and vasoconstriction. It is synthesized in the hypothalamus, stored in the posterior pituitary, and released in response to a decrease in plasma volume or an increase in plasma osmolarity. In the kidneys, vasopressin increases water permeability in the distal tubule and collecting ducts, promoting water retention. In the cardiovascular system, vasopressin constricts blood vessels, raising arterial blood pressure and improving cardiac output (Gordan *et al*, 2015).

The kidneys also produce calcitriol, thrombopoietin, and renin. Renin plays a key role in regulating blood pressure through the renin-angiotensin-aldosterone system (RAAS). RAAS is activated when blood pressure drops, sodium levels decrease, or blood flow slows. Renin converts angiotensinogen to angiotensin I, which is then converted to angiotensin II in the lungs. Angiotensin II increases sodium reabsorption in the kidneys, constricts blood vessels, and stimulates aldosterone release, helping to retain water and regulate blood pressure.

Angiotensin II also has significant cardiovascular effects: it constricts blood vessels, increases systemic blood pressure, promotes platelet aggregation, and stimulates cell growth during myocardial hypertrophy. This makes it a key factor in heart failure and other cardiovascular conditions (al., 2019).

The heart produces two important hormones: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP, released by atrial myocytes, helps reduce blood pressure by blocking catecholamines, inhibiting hypertrophy, and potentially protecting against cardiac fibrosis. BNP, produced by ventricular myocytes in response to stretching, is used clinically to assess heart function, particularly in cases of left ventricular dysfunction or heart failure, as elevated BNP levels indicate poor heart function (Gordan, 2015).

### 1.2.3 Interplay between the cardiovascular system and chromogranins

CgA, CgB, and SgII are proteins released alongside catecholamines by the neuroendocrine system, playing a crucial role in cardiovascular regulation. Derivatives such as vasostatin and catestatin influence vascular tone: vasostatin acts as a vasodilator, while catestatin modulates both vasodilation and vasoconstriction and regulates catecholamine release, preventing excessive sympathetic activation and contributing to blood pressure control.

Chromogranins also exhibit cardioprotective properties by reducing inflammation and fibrosis, protecting the heart under stress or injury. Additionally, they enhance endothelial function by promoting nitric oxide (NO) release and support angiogenesis.

Elevated CgA levels serve as biomarkers for conditions like hypertension and heart failure, and their derivatives are being explored for potential cardiovascular therapies. In summary, chromogranins regulate cardiovascular function, protect against diseases, and offer promising clinical applications for treating heart conditions (Watanabe, 2021).

## 1.3 Interaction between Chromogranins and the Cardiovascular System

### 1.3.1 Chromogranin A and cardiac function regulation

CgA plays a pivotal role in the regulation of cardiac function through its effects on the autonomic nervous system, vascular tone, and myocardial protection. Released by neuroendocrine cells along with catecholamines, CgA acts as a precursor to biologically active peptides such as vasostatin and catestatin, which influence cardiovascular homeostasis (Bartolomucci *et al*, 2011).

Catestatin, a key derivative of CgA, modulates sympathetic nervous system activity by inhibiting excessive catecholamine release, thereby preventing overactivation of the heart and maintaining balanced blood pressure (Troger, 2017).

It also exerts dual effects on vascular tone, promoting vasodilation and countering vasoconstriction, which helps regulate myocardial workload. Additionally, catestatin enhances endothelial function by stimulating nitric oxide (NO) release, contributing to improved vascular health.

CgA and its derivatives exhibit cardioprotective properties by mitigating myocardial inflammation, oxidative stress, and fibrosis, thereby supporting cardiac remodeling and reducing damage during pathological conditions such as hypertension, heart failure, or ischemic injury. Elevated plasma levels of CgA are often associated with cardiovascular diseases, making it a valuable biomarker for diagnosis and prognosis.

Overall, Chromogranin A plays a critical role in cardiac function regulation, acting as both a modulator of autonomic control and a protector against cardiac stress, while its clinical relevance continues to be explored for therapeutic potential in managing cardiovascular diseases (Troger, 2017).

### *1.3.1.1 CgA in regulation of cardiac function*

CgA plays a significant role in regulating cardiac function by influencing autonomic nervous system activity, vascular tone, and myocardial protection.

It is a protein secreted by neuroendocrine cells, often co-released with catecholamines like adrenaline and noradrenaline. CgA acts as a precursor to biologically active peptides, such as catestatin and vasostatin, which are crucial in cardiovascular homeostasis.

Catestatin, a key derivative of CgA, regulates the autonomic nervous system by inhibiting excessive catecholamine release from sympathetic neurons, thus preventing overstimulation of the heart. This action helps maintain balanced heart rate and blood pressure. Additionally, catestatin promotes vasodilation by enhancing nitric oxide (NO) release, which reduces vascular resistance and decreases the workload on the heart (Troger, 2017).

CgA also contributes to cardioprotection by mitigating oxidative stress, inflammation, and fibrosis, processes that are often implicated in cardiac pathologies such as heart failure, ischemia, and hypertension. Furthermore, its derivatives play a role in controlling myocardial remodeling, ensuring that the heart adapts to stress without permanent structural damage.

CgA levels in the bloodstream are often elevated in patients with cardiovascular diseases, making it a potential biomarker for the diagnosis and prognosis of conditions such as heart failure and myocardial infarction. Its role in modulating both autonomic and endothelial functions highlights its therapeutic potential in managing cardiovascular disorders.

CgA is a multifaceted regulator of cardiac function, influencing neural, vascular, and myocardial responses to maintain cardiovascular stability and protect against disease progression (Troger, 2017).

### *1.3.1.2 CgA in regulation of vascular tone*

Chromogranin A plays a pivotal role in the regulation of vascular tone through its influence on endothelial function and smooth muscle activity. As a secreted protein co-released with catecholamines from neuroendocrine cells, CgA is a precursor of biologically active peptides, such as catestatin and vasostatin, which are critical in maintaining vascular homeostasis.

Catestatin, a prominent CgA-derived peptide, is a potent modulator of vascular tone. It promotes vasodilation by stimulating the release of nitric oxide (NO) from endothelial cells.

NO acts on vascular smooth muscle to induce relaxation, thus reducing vascular resistance and blood pressure. This vasodilatory effect is essential for counteracting the vasoconstrictive actions of catecholamines, ensuring balanced vascular responses during stress or heightened sympathetic activity (Watanabe, 2021).

In addition to catestatin, vasostatin, another CgA-derived peptide, contributes to the regulation of vascular tone by inhibiting smooth muscle contraction. This action further supports the maintenance of optimal blood flow and prevents excessive vasoconstriction.

CgA and its derivatives also play a role in mitigating endothelial dysfunction, a key factor in the development of cardiovascular diseases such as hypertension and atherosclerosis. By reducing oxidative stress and inflammation within the vascular endothelium, CgA-derived peptides help preserve the integrity of blood vessels and promote healthy vascular function.

Elevated levels of CgA observed in various cardiovascular disorders suggest its involvement in the pathophysiology of these conditions. Its ability to regulate vascular tone and protect against endothelial dysfunction highlights its potential as a therapeutic target in managing vascular-related diseases (Watanabe, 2021).

CgA regulates vascular tone through its derivatives, such as catestatin and vasostatin, by promoting vasodilation, inhibiting smooth muscle contraction, and protecting endothelial function, thus maintaining vascular homeostasis and preventing pathological vascular responses.

### 1.3.2 Chromogranin B in cardiovascular regulation

CgB, a member of the granin protein family, plays a significant but less explored role in cardiovascular regulation. Like CgA, CgB is stored and co-released with catecholamines from neuroendocrine cells and sympathetic nerve endings. Although its functions are less well understood compared to CgA, emerging evidence highlights its importance in modulating cardiovascular processes, particularly through its influence on the heart and vasculature.

CgB-derived peptides have been shown to contribute to the regulation of cardiac function. These peptides modulate cardiac contractility and heart rate, acting in coordination with other neurohumoral systems. For instance, CgB influences calcium signaling in cardiomyocytes, a critical factor for excitation-contraction coupling in the heart. By regulating intracellular calcium dynamics, CgB can impact myocardial contraction strength and overall cardiac output (Troger, 2017).

In vascular regulation, CgB plays a role in maintaining vascular tone, though its precise mechanisms are less characterized compared to CgA. Evidence suggests that CgB may modulate vascular smooth muscle activity and endothelial function, contributing to vasodilation and blood pressure regulation.

Similar to CgA, CgB-derived peptides may also counteract the vasoconstrictive effects of catecholamines, helping to maintain vascular homeostasis during stress.

Moreover, CgB expression and secretion levels are influenced by physiological and pathological conditions, such as hypertension, heart failure, and other cardiovascular disorders.

Elevated levels of CgB have been observed in patients with certain cardiovascular diseases, suggesting a potential compensatory mechanism or a marker of disease severity. These findings underscore the potential of CgB as a biomarker for cardiovascular conditions and as a therapeutic target (Troger, 2017).

CgB contributes to cardiovascular regulation by influencing cardiac contractility, vascular tone and endothelial function. While less studied than CgA, its emerging role highlights its importance in maintaining cardiovascular homeostasis and its potential relevance in cardiovascular pathology. Further research is needed to fully elucidate the mechanisms and clinical implications of CgB in cardiovascular health and disease.

### 1.3.3 Chromogranins in cardiovascular pathologies

Chromogranins (CgA, CgB, and CgC), originally identified as regulators of catecholamine storage and release in neuroendocrine cells, have emerged as important players in cardiovascular physiology and pathology. Their dysregulation has been implicated in various cardiovascular diseases, including heart failure, hypertension, atherosclerosis, and ischemic heart disease.

CgA is the most extensively studied granin in cardiovascular pathologies. Elevated levels of CgA have been consistently associated with heart failure. In this context, CgA serves as a biomarker, with its circulating levels correlating with the severity of the condition and patient prognosis. CgA-derived peptides, such as vasostatins, catestatin, and serpinin, exhibit both protective and modulatory effects in the cardiovascular system.

For instance, catestatin, a potent inhibitor of catecholamine release, plays a key role in regulating blood pressure by modulating sympathetic tone.

Reduced levels of catestatin have been linked to essential hypertension, suggesting its potential as a therapeutic target (Tota, 2014).

Furthermore, vasostatins exert vasodilatory effects, contributing to vascular homeostasis and opposing excessive vasoconstriction, which is often observed in hypertensive states.

CgB has also been implicated in cardiovascular diseases, although its role is less well understood compared to CgA. Changes in CgB expression and secretion have been reported in heart failure, where it may act as a modulator of calcium signaling in cardiomyocytes. Given the critical role of calcium in excitation-contraction coupling, CgB dysregulation could contribute to the impaired contractility observed in failing hearts (Heidrich, 2018).

In atherosclerosis and ischemic heart disease, chromogranins and their peptides may influence inflammatory processes and endothelial function.

For instance, CgA-derived catestatin has been shown to exert anti-inflammatory and anti-oxidative effects, protecting against endothelial dysfunction and reducing plaque formation. These properties may mitigate the progression of atherosclerosis and reduce the risk of ischemic events.

Additionally, chromogranins may play a role in the pathophysiology of arrhythmias. By modulating autonomic tone, calcium handling, and myocardial excitability, chromogranin-derived peptides could influence the susceptibility to arrhythmias, particularly under conditions of stress or in the presence of structural heart disease (Watanabe *et al*, 2021).

In summary, chromogranins are not only biomarkers of cardiovascular pathologies but also active modulators of the disease process. Their involvement in regulating autonomic tone, vascular reactivity, calcium signaling, and inflammation highlights their multifaceted role in cardiovascular health and disease. Understanding the mechanisms underlying chromogranin dysregulation in cardiovascular pathologies may pave the way for novel therapeutic strategies targeting these molecules and their derived peptides (Watanabe K. T., 2021).

### 1.3.4 Chromogranins as biomarkers in cardiovascular disease

Chromogranins, particularly CgA and CgB, have gained recognition as valuable biomarkers in cardiovascular diseases due to their involvement in regulating various physiological processes, including neuroendocrine secretion, vascular tone, and cardiac function. These proteins, which are secreted by neuroendocrine cells and stored in secretory granules, are cleaved into biologically active peptides that play important roles in cardiovascular regulation.

Elevated levels of chromogranins, especially CgA, have been consistently associated with several cardiovascular conditions, making them potential diagnostic and prognostic tools.

CgA as a Biomarker: CgA is the most studied and well-established chromogranin in the context of cardiovascular disease. It has been linked to a range of conditions, including heart failure, acute coronary syndromes, hypertension, and atherosclerosis (Watanabe *et al*, 2021). CgA is released into the bloodstream during periods of stress or cardiovascular dysfunction, and its elevated levels correlate with disease severity and poor prognosis.

In heart failure, elevated CgA levels have been used as a prognostic marker. High plasma levels of CgA are associated with worse clinical outcomes, including increased mortality and hospitalizations. Additionally, the CgA-derived peptide catestatin, which inhibits catecholamine release, has been found to have a protective role in regulating blood pressure. Reduced levels of catestatin have been observed in patients with hypertension, suggesting its potential as both a diagnostic and therapeutic target (Watanabe K. T., 2021).

CgB and Cardiovascular Diseases: while CgA has garnered the most attention in cardiovascular research, CgB also plays an emerging role. It is involved in modulating calcium signaling and has been linked to cardiac function and disease. Although its clinical significance is still under investigation, changes in CgB levels have been observed in heart failure and other cardiovascular conditions. Its exact role as a biomarker is still not fully understood, but its involvement in regulating myocardial contractility and calcium handling suggests that abnormal CgB expression could be an indicator of cardiovascular dysfunction (Røsjø S. F., 2010).

CgA-Derived Peptides in Cardiovascular Disease: the peptides derived from chromogranins, such as catestatin, vasostatin, and pancreastatin, have shown promise as biomarkers due to their specific biological effects. Catestatin, for instance, not only inhibits catecholamine release but also exhibits anti-inflammatory and vasodilatory properties, contributing to vascular homeostasis. Changes in the balance of these peptides can reflect shifts in autonomic regulation and vascular health, which are critical in cardiovascular disease progression (Watanabe, 2021).

In conditions like atherosclerosis and ischemic heart disease, CgA-derived peptides such as catestatin can act protectively by reducing oxidative stress and inflammation, thus slowing disease progression. Furthermore, vasostatins, which are also derived from CgA, can counteract excessive vasoconstriction, providing a potential therapeutic pathway for controlling hypertension and preventing cardiovascular events.

Chromogranins, particularly CgA and its derived peptides, hold significant promise as biomarkers in cardiovascular disease.

Elevated CgA levels are indicative of stress, disease severity, and poor prognosis in conditions such as heart failure and hypertension. The ability to measure CgA, CgB, and their peptides in blood provides a potential non-invasive method for diagnosing cardiovascular diseases and predicting outcomes.

Understanding the mechanisms by which chromogranins influence cardiovascular pathophysiology may lead to new therapeutic approaches, targeting these proteins or their peptides to improve patient outcomes in cardiovascular diseases (D'Amico, 2014).

## 1.4 Chromogranins as potential therapeutic targets in cardiovascular disease

### 1.4.1 Rationale for targeting chromogranins in therapy

One of the most promising therapeutic approaches centers around catestatin, a peptide derived from CgA. Catestatin inhibits the release of catecholamines from the adrenal medulla and sympathetic nerve terminals, helping to regulate blood pressure and protect against hypertensive damage. In patients with hypertension, reduced levels of catestatin have been observed, highlighting its protective role in maintaining vascular tone and preventing excessive vasoconstriction.

Enhancing catestatin activity or mimicking its effects could serve as a potential therapeutic strategy for controlling high blood pressure and preventing cardiovascular events linked to hypertension (Mahata, 2018).

In heart failure, elevated levels of CgA have been associated with a poor prognosis, and reducing these levels may help manage the condition. Modulating the release or processing of CgA could offer a way to regulate cardiovascular stress responses, alleviate symptoms, and improve patient outcomes. Pharmacologically targeting CgA or its peptides could potentially restore more balanced neuroendocrine signaling, which is crucial for managing heart failure effectively (Tota, 2014).

Vasostatin, another peptide derived from CgA, is known for its vasodilatory effects and ability to inhibit vasoconstriction. This peptide could be used to treat conditions such as atherosclerosis or ischemic heart disease, where excessive vasoconstriction and vascular stiffness are key contributors to disease progression.

Targeting vasostatin pathways could help reduce vascular resistance, improve blood flow, and protect against the development of arterial plaque.

Endothelial dysfunction, a critical factor in the development of atherosclerosis and other cardiovascular diseases, could also be addressed by chromogranin-derived peptides.

These peptides may have the potential to modulate inflammation, oxidative stress, and endothelial cell function, offering protection against vascular damage. For instance, the ability of catestatin and vasostatin to reduce oxidative stress and inflammation suggests that they could serve as therapeutic agents to restore endothelial function and prevent further cardiovascular damage (Tota, 2014).

However, targeting chromogranins as a therapeutic strategy is not without challenges. While their roles in cardiovascular regulation are becoming clearer, the exact mechanisms through which chromogranins influence cardiovascular function are not fully understood.

Further research is needed to identify the precise contributions of CgA, CgB, and their derived peptides in different cardiovascular diseases. Additionally, developing therapies that selectively modulate chromogranin expression or activity without causing unintended effects on other systems is crucial.

The complex interaction between chromogranins and other neurohormonal pathways must be carefully considered to avoid potential adverse outcomes (Bartolomucci *et al*, 2011).

In conclusion, chromogranins, particularly CgA and CgB, hold significant therapeutic potential in cardiovascular diseases due to their roles in regulating neuroendocrine signaling, vascular tone, and myocardial function. By modulating the activity of chromogranin-derived peptides such as catestatin and vasostatin, it may be possible to improve blood pressure regulation, reduce inflammation, protect the vasculature, and manage conditions like heart failure and hypertension.

Nevertheless, further research is required to fully understand the mechanisms by which chromogranins affect cardiovascular health and to develop safe and effective therapies targeting these proteins. As our understanding of chromogranins in cardiovascular disease deepens, they may become integral components of future treatment strategies aimed at improving patient outcomes (Bartolomucci, 2011).

### 1.4.2 Chromogranin modulation: Targeted expression in cardiovascular tissues

Chromogranin modulation through targeted expression in cardiovascular tissues is a promising area of research with potential therapeutic implications for various cardiovascular diseases. Chromogranins, especially CgA and CgB, are key regulatory proteins in the cardiovascular system, playing a role in controlling neuroendocrine secretion, vascular tone, and myocardial function.

By modulating their expression and activity within specific cardiovascular tissues, it may be possible to develop targeted treatments for conditions such as hypertension, heart failure, atherosclerosis, and ischemic heart disease (Troger, 2017).

One of the most important aspects of chromogranin modulation is the ability to control the synthesis and release of their derived peptides, such as catestatin and vasostatin, which have vasodilatory, anti-inflammatory, and cardioprotective effects. These peptides act on the cardiovascular system by regulating blood pressure, reducing oxidative stress, and protecting the heart and blood vessels from damage. Targeting chromogranin expression in specific cardiovascular tissues could provide a more localized and precise therapeutic effect, minimizing systemic side effects and enhancing the efficacy of treatment.

The potential for targeted expression of chromogranins in cardiovascular tissues lies in the use of gene therapy and advanced delivery systems.

By utilizing vectors or nanoparticles to deliver chromogranin genes directly to cardiovascular cells, researchers could increase or decrease the production of chromogranin proteins or their peptides where they are most needed. For example, increasing the expression of CgA in the heart could lead to higher levels of catestatin and vasostatin, which would help regulate blood pressure, improve endothelial function, and protect against myocardial injury. On the other hand, reducing the expression of CgA in the heart could be beneficial in conditions where its overproduction contributes to disease progression, such as in heart failure (Loh YP, 2012).

The cardiovascular system is highly responsive to neurohormonal signaling, and chromogranins are central to this regulation. By controlling chromogranin expression in specific tissues like the heart, arteries, and veins, it may be possible to fine-tune the neurohormonal balance, improving cardiovascular function. For example, chromogranin-derived peptides like catestatin can inhibit the release of catecholamines, which are crucial in maintaining vascular tone and heart rate.

In a hypertensive state, where catecholamine levels are elevated, increasing the expression of catestatin in vascular smooth muscle cells could help counteract excessive vasoconstriction and lower blood pressure.

In addition, chromogranins could be used to target specific mechanisms involved in vascular remodeling, such as inflammation and oxidative stress. In diseases like atherosclerosis, where oxidative damage and inflammation contribute to plaque formation and arterial stiffness, modulating chromogranin expression could reduce these harmful effects (Angelone, 2012).

For instance, vasostatin has vasodilatory and anti-inflammatory properties, making it an attractive candidate for treating conditions characterized by excessive vascular constriction and inflammation (Troger, 2017).

Despite the potential benefits, several challenges remain in translating chromogranin modulation into clinical practice. The complexity of chromogranin biology and its interactions with other neurohormonal systems must be thoroughly understood to ensure that targeted therapies do not have unintended consequences. Additionally, the delivery mechanisms for gene therapy or peptide-based treatments must be optimized to achieve efficient and sustained expression within the desired cardiovascular tissues. The development of safe and effective delivery systems will be crucial for ensuring that chromogranin-based therapies can be successfully translated into clinical applications.

In conclusion, targeted modulation of chromogranin expression in cardiovascular tissues represents a novel approach to treating cardiovascular diseases. By regulating the production and release of chromogranin-derived peptides like catestatin and vasostatin, it may be possible to improve blood pressure regulation, reduce inflammation, protect against vascular damage, and support heart function (Watanabe K. T., 2021).

While more research is needed to better understand the mechanisms of chromogranin regulation and to develop effective delivery systems, this approach holds great promise for the future of cardiovascular disease therapy.

# 2. AIM OF THE STUDY

The main objective of this study is to investigate the effects of CgB on human umbilical vein endothelial cells (HUVECs) under both physiological and oxidative stress conditions. Specifically, the study is focused on key cellular parameters, including cell viability, mitochondrial membrane potential, reactive oxygen species (ROS) production, glutathione (GSH) levels, nitric oxide (NO) release, and intracellular calcium concentration.

A crucial aspect of this investigation was the exploration of the molecular mechanisms underlying CgB's effects on endothelial cells. To this end, specific intracellular signaling pathways, such as Pi3K/Akt, MEK1/2/ERK1/2, AMPK, CaMKII, and PKA, were analyzed to understand their role in modulating CgB's actions.

The goal is to expand current knowledge regarding the potential of CgB as a protective agent against endothelial dysfunction, with possible implications for cardiovascular disease prevention and treatment.

# 3. MATERIALS AND METHODS

## 3.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultivated in DMEM (Dulbecco's Modified Eagle Medium) enriched with 2mM L-glutamine, 1500 mg/L sodium bicarbonate and supplemented with 0,1 mg/mL heparin, 1% penicillin, 1% streptomycin and 10% fetal bovine serum (FBS). The cell cultures were maintained at 37°C in an atmosphere containing $CO_2$. For the experimental assays, 1 x $10^4$ cells were seeded in 96 well plates with DMEM containing 0% FBS, 1% penicillin-streptomycin-glutamine and no phenol red for a period of 4-6 hours. Each experiment was conducted in triplicate and repeated three times using different HUVEC pools (Grossini E, 2024).

## 3.2 Cell viability measurement

Cell viability in HUVECs was assessed using the MTT assay, specifically with 1% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide dye, following established protocols. During the initial phase, a dose-response and time-course study was conducted to determine the optimal concentration and stimulation period for CgB. HUVECs were treated with fully synthetic human CgB at concentrations of 1 pM, 100 pM, 10 nM, and 100 nM for durations of 1, 5, 15, 30 and 60 minutes. The experiments were also performed in the presence of hydrogen peroxide ($H_2O_2$, 200 μM for 30 minutes) and N-acetylcysteine (NAC, 200 μM for 30 minutes), which served as a positive control. In the next phase, the selected CgB concentration and timing were tested with or without specific pathway inhibitors, including H89 (PKA inhibitor), dorsomorphin (AMPK inhibitor), wortmannin (pan-Pi3K inhibitor), UO126 (MEK1/2 inhibitor) and KN93 (CaMKII inhibitor), all at 10 nM and dissolved in DMSO. These inhibitors were co-administered with CgB.

In particular, those inhibitors are involved in:

- L-NAME, NO Synthase Inhibitor: it's a non-selective inhibitor of NO synthase (NOS), the enzyme responsible for synthesizing NO from L-arginine. The use of L-NAME is important to assess whether NO production in response to CgB depends on NOS activity;
- H89 (protein kinase A inhibitor, PKA): it's a competitive ATP inhibitor of PKA. PKA is involved in regulating endothelial NOS and phosphorylating proteins that influence NO production;
- Dorsomorphin (AMPK pathway inhibitor): AMPK (AMP-activated protein kinase) is a regulator of cellular metabolism and can influence endothelial NOS activity;
- Wortmannin (PI3K inhibitor): PI3K (phosphatidylinositol 3-kinase) is involved in intracellular signaling that regulates cell survival and NO production via eNOS phosphorylation;
- UO126 (MAPK/ERK pathway inhibitor): The MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway is involved in proliferation and response to extracellular stimuli;
- KN93 (CaMKII inhibitor): CaMKII (calmodulin-dependent protein kinase II) is a calcium/calmodulin-regulated kinase that plays a crucial role in eNOS regulation.

Following each stimulation, the medium was replaced with fresh culture medium (devoid of phenol red and FBS). The MTT dye was then added and the cells were incubated at 37 °C for 2 hours. After incubation, the medium was discarded and replaced with an MTT solubilization solution (dimethyl sulfoxide) to dissolve the resulting formazan crystals. Absorbance was measured at 570 nm using a VICTOR™ X Multilabel Plate Reader.
Cell viability was compared to untreated control cells (non-treated cells; DMSO only), which were set as the 100% baseline (Grossini E, 2025 a) (Grossini E, 2023 d).

The assay is based on the yellow tetrazolium salt TT to purple formazan crystal by active cells. This cellular reduction process uses the pyridine nucleotide cofactors NADH and NADPH. The formed formazan crystals are dissolved, and the resulting colored solution is measured using a multiwell spectrophotometer (Fig. 6). Tetrazolium salt (such as MTT) is useful for assaying the quantification of viable cells, because only metabolically active cells are able to cleave it to form a formazan dye (Grossini E, 2023 d).

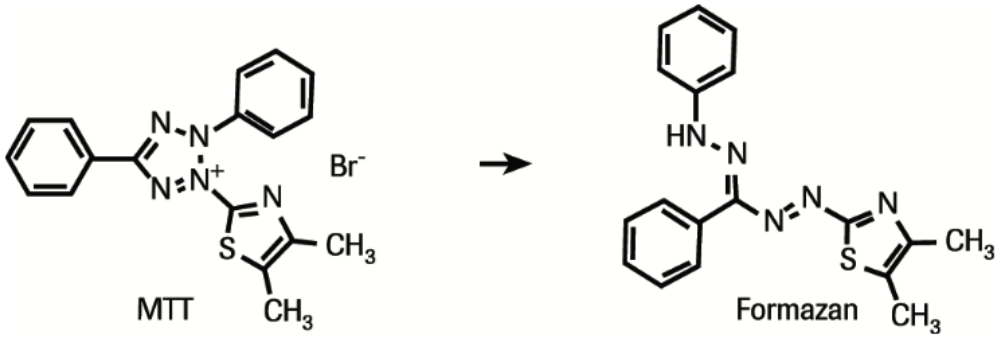



**Figure 6**: Metabolization of MTT to a formazan salt by viable cells (Riss, 2013).

## 3.3 NO release measurement

NO production in HUVEC supernatants was evaluated using the Griess assay, following previously established methods.

This system is based on the chemical reaction which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions (Fig. 7). Sulfanilamide and NED compete for nitrite in the Griess reaction (Fig.7).

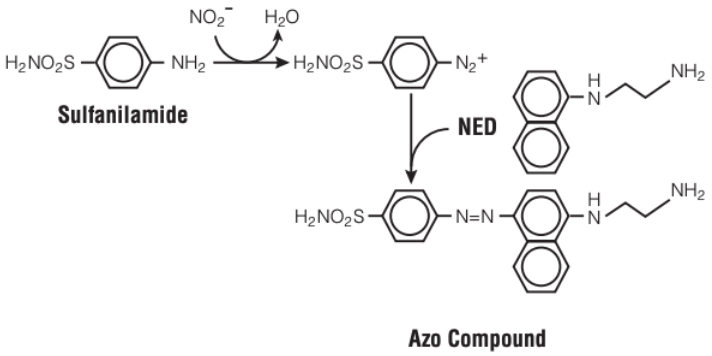



**Figure 7:** Chemical reactions involved in the measurement of $NO_2^-$ using the Griess Reagent System (Sigma-Aldrich, 2021).

To determine the appropriate concentration and stimulation duration for CgB, a preliminary phase was conducted similar to the protocol used for cell viability assessments.

Some of these initial experiments were also performed in the presence of hydrogen peroxide ($H_2O_2$), as done for cell viability and as previously executed (Grossini E, 2024) (Grossini E, 2025 a) (Grossini E, 2023 b) (Grossini E, 2023 d).

In the subsequent experimental phase, HUVECs were treated with the selected CgB concentration and exposure time, either alone or in combination with various agonist or antagonist.

These included the β-adrenergic receptor agonist isoproterenol (10 nM), the β2-adrenergic receptor antagonist butoxamine (10 nM, dissolved in DMSO), the α-adrenergic receptor agonist phenylephrine (10 nM, dissolved in DMSO), and the α-adrenergic receptor antagonist phentolamine (10 nM, dissolved in DMSO). Additional treatments involved the muscarinic receptor antagonist atropine (10 nM, dissolved in DMSO), the NOS inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10 mM, dissolved in DMSO), as well as, the pathway inhibitors H89, dorsomorphin, wortmannin, UO126, and KN93. All these compounds were co-administered with CgB. Acetylcholine (10 μM, dissolved in DMSO) served as a positive control (Grossini E, 2024).

The combined use of those inhibitors is useful to provide a clearer picture of the mechanism of action of CgB in HUVECs. If NO production is inhibited or increased by specific blockers/agonists, it means that CgB activates those pathways to exert its biological effect. Conversely, if NO production remains unchanged, the involvement of that specific signaling pathway can be ruled out. These experiments are essential to understanding the role of CgB in regulating endothelial function and NO release, with potential implications for cardiovascular physiology and the development of new therapeutic strategies for cardiovascular diseases (Grossini E, 2024).

Following each treatment, NO levels in the supernatants were assessed by mixing the samples with an equal volume of Griess reagent. The sulfanilamide and NED solutions were allowed to reach room temperature for 15–30 minutes before use. Subsequently, 50 μL of each sample was transferred into well plates in triplicate. An equal volume (50 μL) of sulfanilamide solution was added to all wells using a multichannel pipette.

After a 10-minute incubation in the dark at room temperature, 50 μL of NED solution was added to each well. The absorbance at 570 nm was then measured using a VICTOR™ X Multilabel Plate Reader. NO production was quantified by referencing a nitrate standard curve.

The resulting values corresponded to the amount of NO (in µM) produced in each sample containing 1.5 µg of protein. NO release from HUVECs was compared to untreated control cells (DMSO only), with control values normalized to 1.

## 1.4 Mitochondrial membrane potential measurement

The mitochondrial membrane potential (ΔΨM) can be assessed using various cationic fluorescent dyes, including JC-1, which allows differentiation between healthy and apoptotic cells.

JC-1 is a lipophilic, cationic dye that, under normal conditions, enters negatively charged mitochondria, forming red J aggregates. In apoptotic or dysfunctional cells, however, the membrane potential decreases, preventing the formation of aggregates and maintaining green fluorescence (Fig. 8).

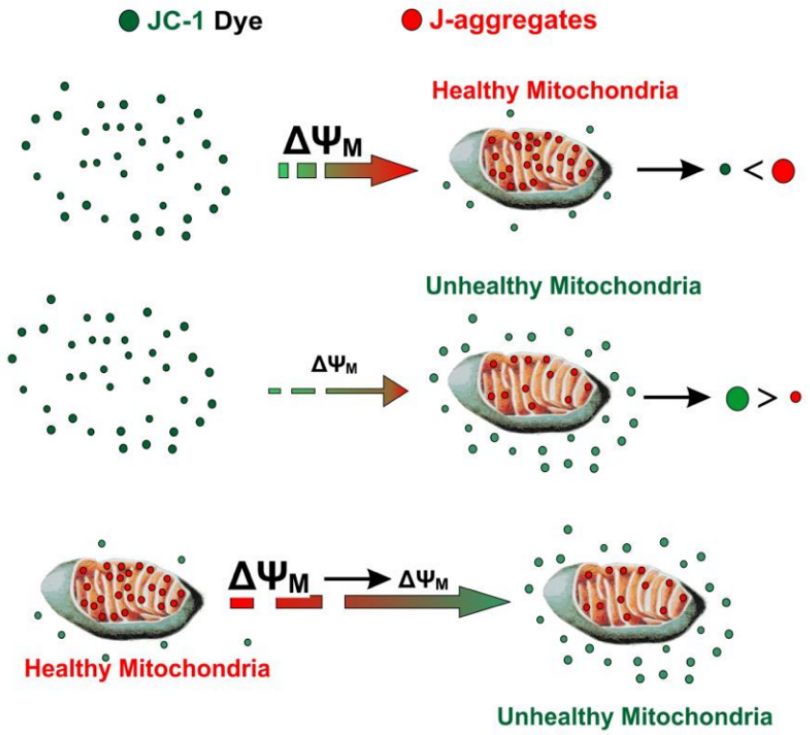



**Figure 8**: Illustration depicting JC-1 entry into the mitochondria and the generation of J aggregate. JC-1, a cationic carbocyanine dye (green) exhibits potential-dependent accumulation in mitochondria where it starts forming J aggregates (red); upon depolarization, it remains as monomer showing green fluorescence (Sivandzade, 2019).

The red-to-green fluorescence ratio is a direct indicator of mitochondrial polarization: a high ratio indicates healthy mitochondria (high ΔΨM), while a reduced ratio suggests mitochondrial depolarization (Sivandzade, 2019).

To assess the mitochondrial membrane potential of HUVECs, the JC-1 assay was employed. The methods were the same used previously to evaluate mitochondrial membrane potential in HUVECs (Grossini E, 2023 d) (Grossini E, 2025 a).

HUVECs were treated with CgB at concentrations of 1 pM, 100 pM, 10 nM, and 100 nM for the duration (30 min) identified in the preliminary phase. These treatments were conducted both with and without hydrogen peroxide ($H_2O_2$, 200 μM for 30 minutes), following the same protocol used for cell viability and NO release assessments. In the subsequent phase, HUVECs were again treated with the selected CgB concentration and timing, either alone or combined with pathway inhibitors. These included H89, dorsomorphin, wortmannin, UO126, and KN93 (all at 10 nM, dissolved in DMSO), which were co-administered with CgB (Grossini E, 2024).

The medium was discarded, and cells were incubated for 15 minutes at 37 °C with JC-1 dye (diluted 1× in Assay Buffer 1×). The cells were then washed twice with Assay Buffer 1×. Fluorescence was measured using a spectrophotometer (VICTOR™ X Multilabel Plate Reader) by recording red fluorescence (excitation 550 nm/emission 600 nm) and green fluorescence (excitation 485 nm/emission 535 nm).

The mitochondrial membrane potential in treated cells was compared to that of untreated control cells (DMSO only), with the control value set to 100 for normalization.

## 3.5 ROS Release measurement

Reactive oxygen species (ROS) include a number of reactive molecules and free radicals, derived from molecular oxygen, that damage DNA and oxidize proteins and lipids (lipid peroxidation). The most common ROS include superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen ($O_2$). The molecules are produced during the electron transport of mitochondrial aerobic respiration or by oxidoreductase enzymes and metal catalysed oxidation. The conversion of 2,7-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) into 2,7-dichlorodihydrofluorescein (DCF) was used to evaluate ROS release, as previously performed in the same cell line (Fig. 9) (Grossini E, 2023 d) (Grossini E, 2025 a) (Grossini E, 2025 c).

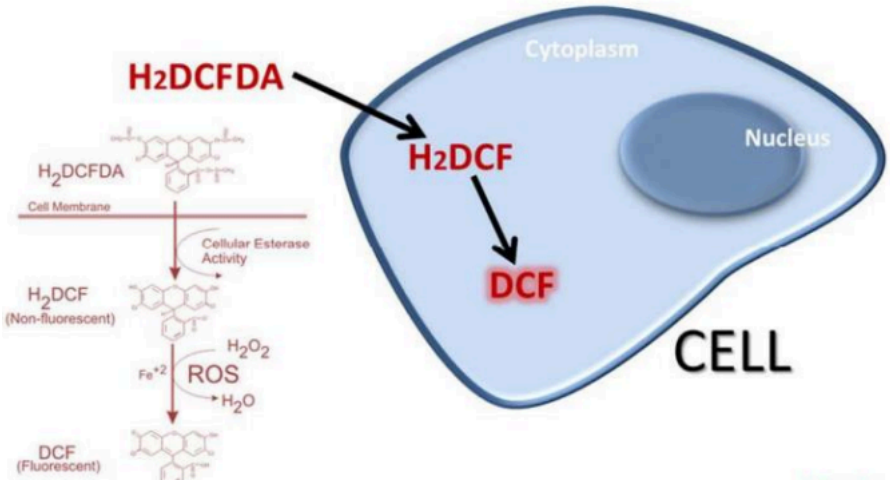



**Figure 9:** Formation of DCF compound from the reaction of $H_2$DCFDA plus ROS (Sivandzade, 2019).

HUVECs were stimulated with 1 pM, 100 pM, 10 nM, and 100 nM of CgB, according to the timing established during the preliminary phase, in the presence of $H_2O_2$ (200 µM for 30 minutes). NAC, at a concentration of 200 µM with a stimulation time of 30 minutes, was used as a positive control.

After each stimulation, cells were stained with 10 µM of $H_2$DCFDA for 20 minutes at 37 °C. The fluorescence intensity of DCF was measured using a fluorescence plate reader (VICTOR™ X Multilabel Plate Reader) with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Results were expressed as DCF fluorescence intensity.

## 3.6 GSH measurement

Glutathione (GSH) is a tripeptide used as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides.

In HUVECs, the measurement of GSH was carried out using a specific kit from Cayman Chemical. The cells underwent the same treatment process as for ROS release assessment.

After treatment, the cells were lysed with 50 mM of 2-(N-morpholino) ethanesulfonic acid (GSH MES Buffer) using a rubber policeman. The resulting cell pellet was centrifuged at 10,000× g for 15 minutes at 4 °C (Grossini E, 2021) (Grossini E, 2024).

The supernatant obtained after centrifugation was mixed with an equal volume of metaphosphoric acid (final concentration 5%) for 5 minutes and then centrifuged again at 2000× g for at least 2 minutes. The resulting supernatant was collected and supplemented with 50 µL of a 4 M triethanolamine per milliliter.

Subsequently, 50 µL of each sample was transferred to a 96-well plate, where GSH levels were measured according to the manufacturer's guidelines using a spectrophotometer (VICTOR$^{TM}$ X Multilabel Plate Reader) with excitation and emission wavelengths of 405–414 nm. GSH concentration was reported as nanomoles in samples containing 1.5 mg of protein per mL.

## 3.7 Measurement of $[Ca^{2+}]c$ by Fura-2 Fluorescence

Intracellular calcium concentration ($[Ca^{2+}]c$) is one of the main second messengers in cells and plays an essential role in numerous physiological processes. Its measurement is crucial for understanding cellular function, signal transduction and cellular homeostasis. Alterations in the concentration of $[Ca^{2+}]c$ are often associated to cardiovascular disease, as calcium regulates endothelial function and vasodilatation. An imbalance can contribute to hypertension and atherosclerosis.

To measure intracellular calcium concentration ($[Ca^{2+}]c$), HUVECs were cultured until they reached confluence. The cells were then washed twice with sterile 1× phosphate-buffered saline (PBS) and incubated with 5 µM of fura-2/acetoxymethyl (AM) ester in DMEM containing 10% FBS and no phenol red for 30 minutes in the dark. Following additional washes with DMEM, the coverslips were placed in a thermostatted quartz cuvette inside an agitation system set at 37 °C. The calcium measurement was performed using a Hitachi F-4500 Fluorescence Spectrometer for 300 seconds, with an excitation wavelength of 340 nm and an emission wavelength of 510 nm (Grossini E, 2024).

During the preliminary phase, Fura-2/AM-loaded HUVECs were stimulated with CgB at concentrations of 1 pM, 100 pM, 10 nM, and 100 nM according to the established stimulation timing, either in the presence or absence of $H_2O_2$ (200 µM for 30 minutes).

The effects of CgB were compared with those triggered by ATP (10 μM). Additionally, some experiments were conducted with or without calcium in the incubation medium, achieved by adding 50 mM ethylene glycol tetraacetic acid (EGTA).

Further experiments involved administering CgB at the previously defined concentration and timing, either alone or in combination with isoproterenol, butoxamine, H89, and KN93 (all at 10 nM, dissolved in DMSO).

The intracellular calcium concentration ($[Ca^{2+}]c$) was calculated using the following equation: $(Ca^{2+}) = Kd\ ((R-Rmin)/(Rmax-R))$.

The dissociation constant (Kd) of fura-2/AM for calcium was set at 224. The minimum (Rmin) and maximum (Rmax) fluorescence ratio values were determined under calcium-free conditions (0.1 M EGTA) and calcium-saturated conditions, respectively. The recorded fluorescence intensities were adjusted to account for cell autofluorescence at the corresponding wavelengths.

## 3.8 Statistical analysis

All data are expressed as the median and range, obtained from three independent experiments conducted on different pools of HUVECs, each with triplicate readings. Differences between two or more groups were analyzed using the Mann–Whitney test and the Kruskal–Wallis test, followed by Dunn's post hoc test for multiple comparisons. The non-parametric Mann–Kendall trend test was used to assess the trend of values over time. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed, and graphs were generated using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, USA) and STATA v.17 (StataCorp, 2021 Statistical Software: Release 17, College Station, TX, USA).

# 4. RESULTS

As illustrated in Fig. 10, the dose-response and time-course experiments revealed that HUVECs viability under physiological conditions increased progressively with CgB concentrations ranging from 1 pM to 10 nM, where a plateau effect was reached. Likewise, 30 minutes of CgB stimulation resulted in the maximum enhancement of cell viability (Fig. 10 A,B). Moreover, the same duration of stimulation was sufficient to mitigate the detrimental effects of hydrogen peroxide ($H_2O_2$) on HUVECs, also in a dose-dependent fashion (Fig. 10C) (Grossini E, 2024).

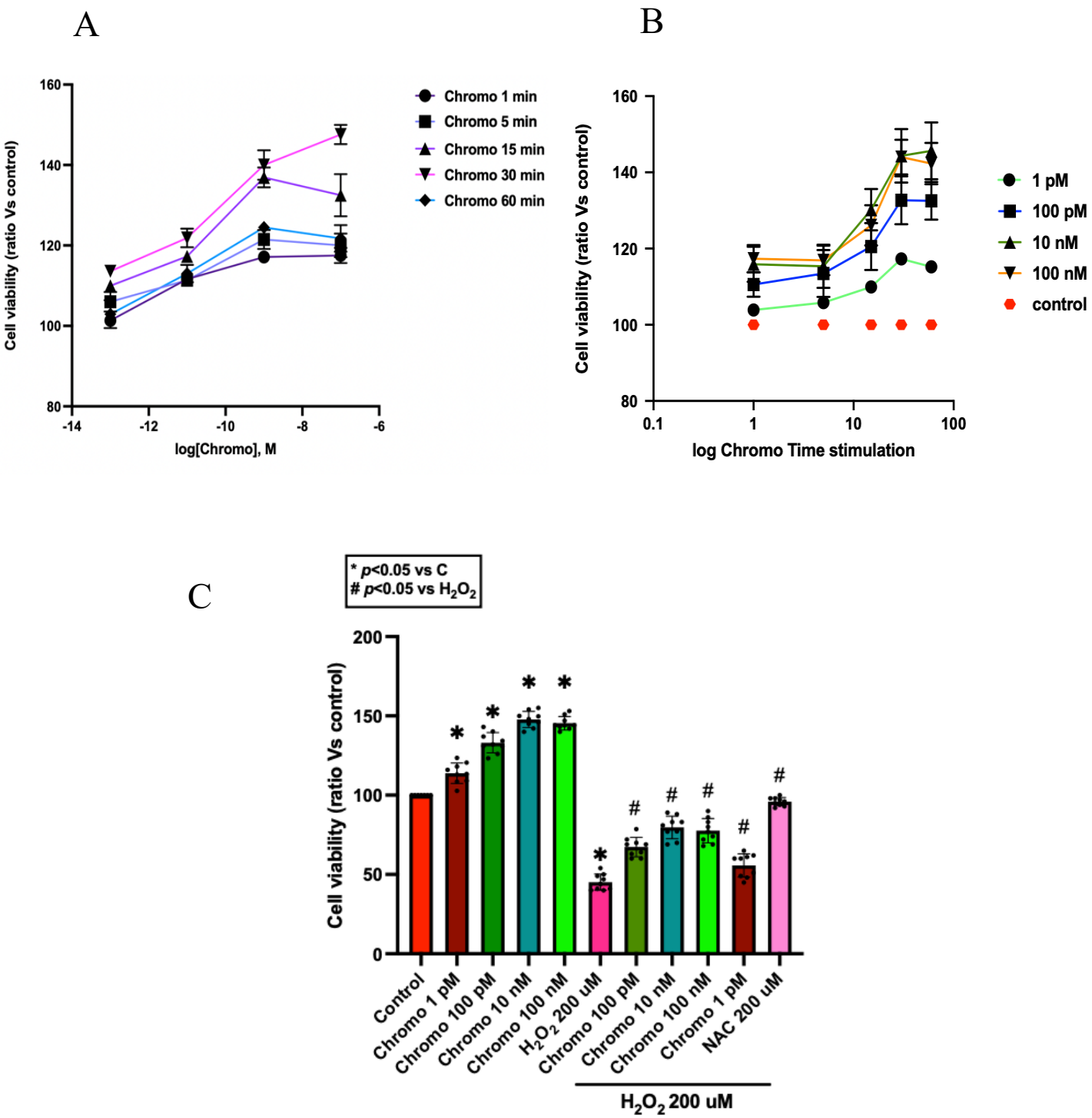



**Figure 10**: Impact of chromogranin B on HUVEC viability. Panel (A) illustrates the dose-response analysis, while Panel (B) displays the time-course experiment, with stimulation durations of 1, 5, 15, 30, and 60 minutes. Panel (C) shows the effects of chromogranin B with or without the presence of hydrogen peroxide ($H_2O_2$). Data are presented as medians with ranges, based on three independent experiments using different HUVECs donor pools, each measured in triplicate. "C" represents the untreated control cells (vehicle only, DMSO). "Chromo" refers to chromogranin B, and "NAC" denotes N-acetylcysteine. For Panels (A) and (B), statistical comparisons between different concentrations of chromogranin B at each time point were conducted using the Kruskal–Wallis test, followed by Dunn's multiple comparisons post-test. In Panel (C), the Mann–Whitney test was used to compare the effects of different chromogranin B concentrations to the control group and to the $H_2O_2$-treated group (symbols "*" and "#" reflect these comparisons, respectively). A p-value < 0.05 was considered statistically significant (Grossini E, 2024).

Stimulation with CgB under physiological conditions also promoted NO production in a concentration-dependent manner (Fig. 11). Similar to the viability data, the peak NO release occurred at 10 nM CgB. The time-course analysis confirmed that 30 minutes of exposure was optimal for NO release (Fig. 11A).

Interestingly, under oxidative stress conditions, where NO levels were inherently elevated, 30-minute stimulation with CgB led to a dose-dependent decrease in NO production, with the maximum reduction observed at 10 nM (Fig. 11C) (Grossini E, 2024).

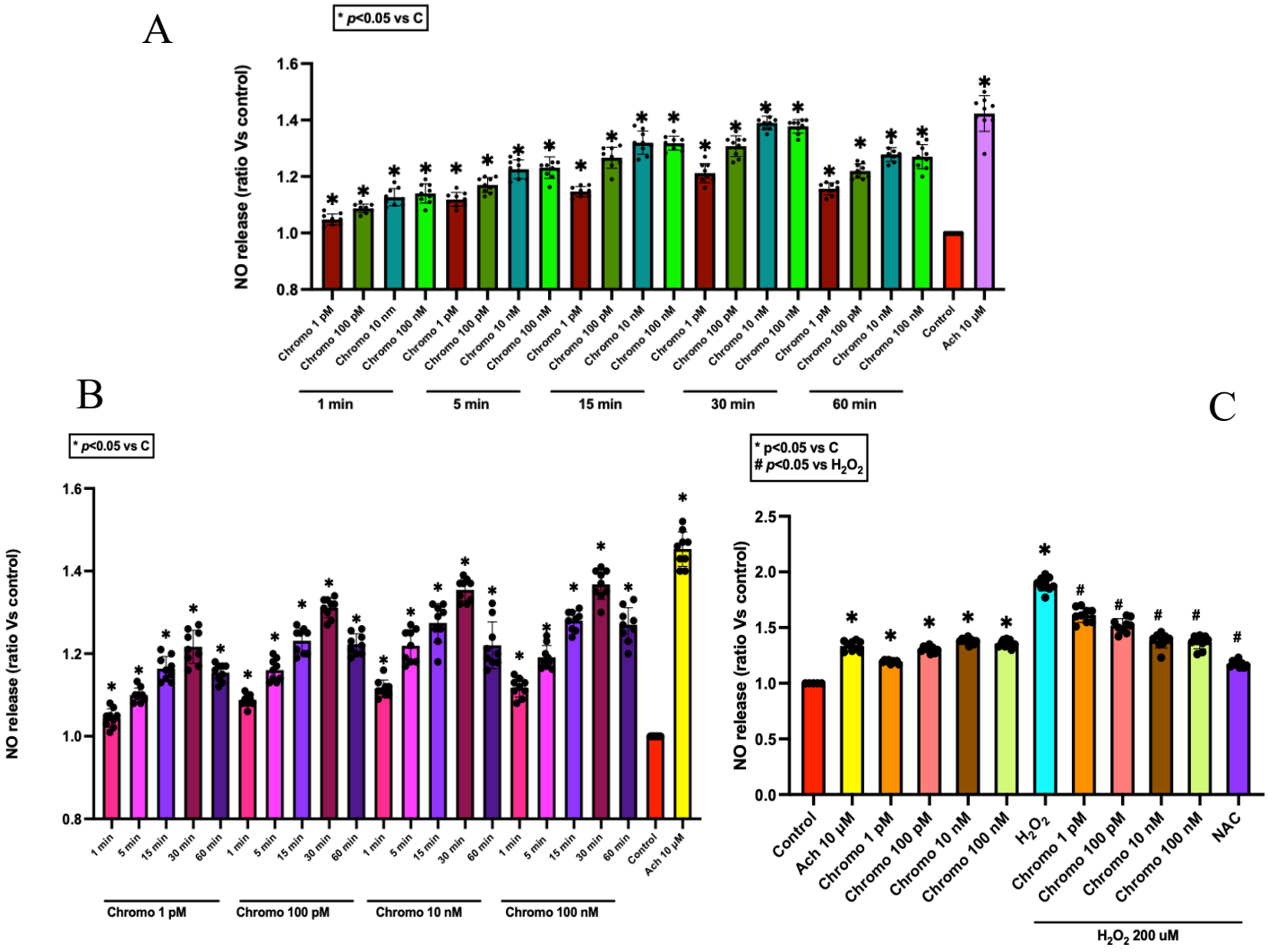



**Figure 11**: Influence of chromogranin B on nitric oxide (NO) production in HUVECs. Panel (A) presents the dose-response assessment, while Panel (B) shows the temporal analysis of NO release. Panel (C) illustrates the impact of chromogranin B on NO release in the presence or absence of hydrogen peroxide ($H_2O_2$). "Ach" refers to acetylcholine, while all other abbreviations are consistent with those used in Figure 10. In Panels (A) and (B), statistical comparisons between various concentrations of chromogranin B at each time point were carried out using the Kruskal–Wallis test, followed by Dunn's post hoc multiple comparisons test. For Panel (C), the Mann–Whitney U test was employed to assess differences between chromogranin B concentrations, comparing them to untreated controls and $H_2O_2$-treated samples, respectively (symbols "*" and "#" refer to these comparisons). A p-value < 0.05 was used to determine statistical significance (Grossini E, 2024).

The cytoprotective role of CgB was further validated by assessing the mitochondrial membrane potential. As shown in Fig. 12A, in HUVECs maintained in physiological conditions, 30 minutes of CgB stimulation significantly increased mitochondrial membrane potential, again in a dose-dependent manner, up to 10 nM. In the context of oxidative stress induced by $H_2O_2$, CgB demonstrated a protective role, mitigating the damaging effects of the peroxidative insult. As illustrated in Figs. 12B and 12C, CgB lowered the elevated levels of ROS triggered by $H_2O_2$ and restored GSH levels, which had been diminished by $H_2O_2$ (Grossini E, 2024).

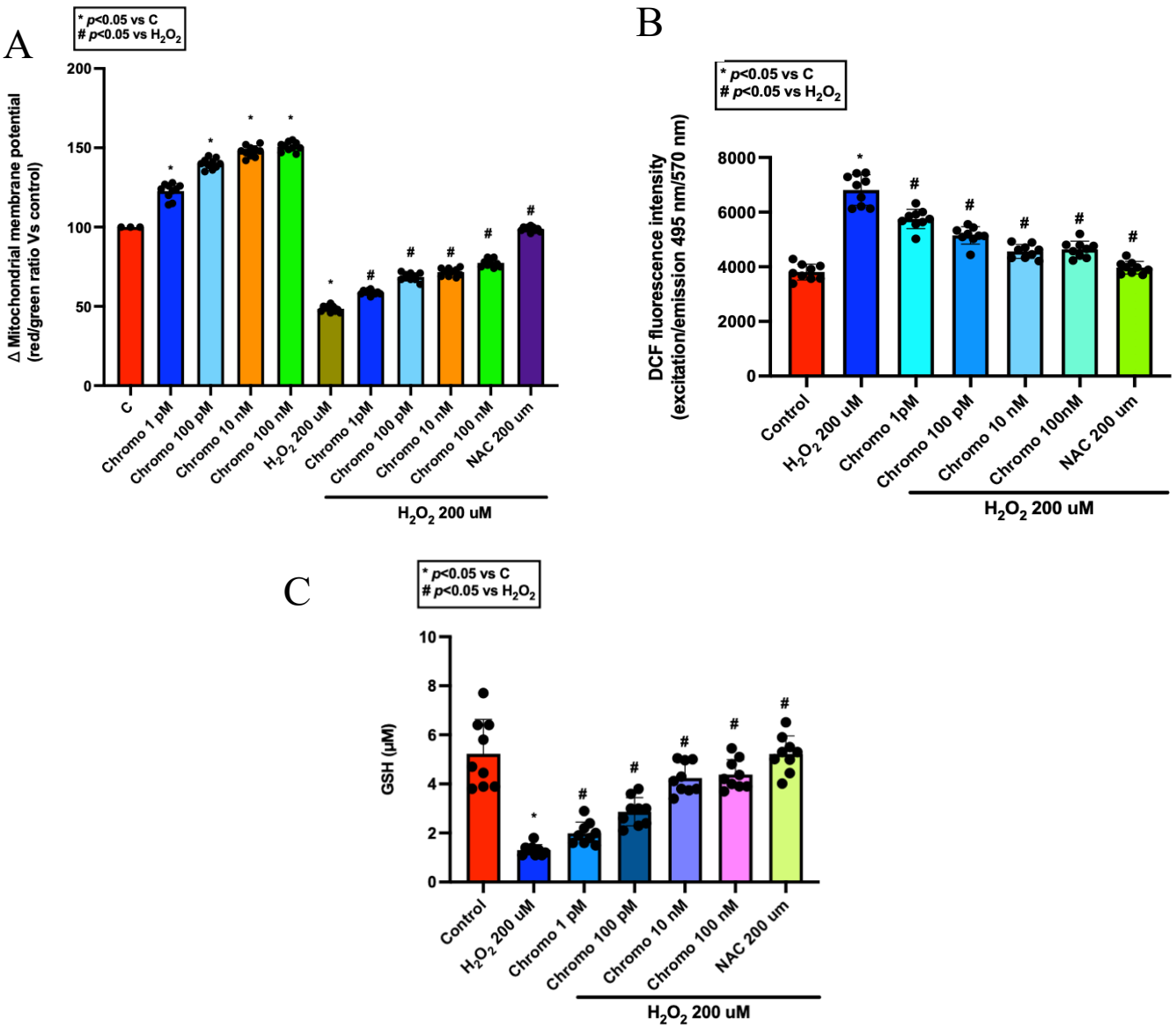



**Figure 12**: Chromogranin B effects on mitochondrial function and oxidant/antioxidant in HUVECs. Panel (A) displays the impact on mitochondrial membrane potential, Panel (B) shows reactive oxygen species (ROS) production, and Panel (C) illustrates glutathione (GSH) levels. ROS levels are represented by DCF fluorescence intensity. In Panels (A–C), the Mann–Whitney U test was applied to evaluate differences between various concentrations of chromogranin B, with comparisons made against untreated controls and $H_2O_2$-treated samples. A p-value lower than 0.05 was considered statistically significant (Grossini E, 2024).

To explore the intracellular signaling mechanisms underlying CgB's action on HUVECs under normal conditions, further experiments were conducted using specific inhibitors targeting key pathways: PKA (H89), AMPK (dorsomorphin), pan-PI3K (wortmannin), MEK1/2 (U0126), and CaMKII (KN93), each applied at 10 nM.

As shown in Fig. 13A, inhibition of PKA, AMPK, and CaMKII significantly reduced the beneficial effects of CgB on cell viability. Moreover, the inhibition of ERK1/2 and Akt signaling completely abolished CgB’s effect (Fig. 13A). Comparable outcomes were also noted in the evaluation of mitochondrial membrane potential (Fig. 13B).

In HUVECs exposed to oxidative stress, the inhibition of PKA, AMPK, MEK1/2, and pan-PI3K pathways blocked the protective effects of CgB on cell viability (Fig. 13A). When CaMKII was inhibited, a partial reduction in CgB’s protective action was observed.

For the mitochondrial membrane potential, MEK1/2 and pan-PI3K inhibitors fully suppressed the beneficial effect of CgB, whereas inhibitors of PKA, AMPK, and CaMKII led to a partial attenuation of this response (Fig. 13B) (Grossini E, 2024).

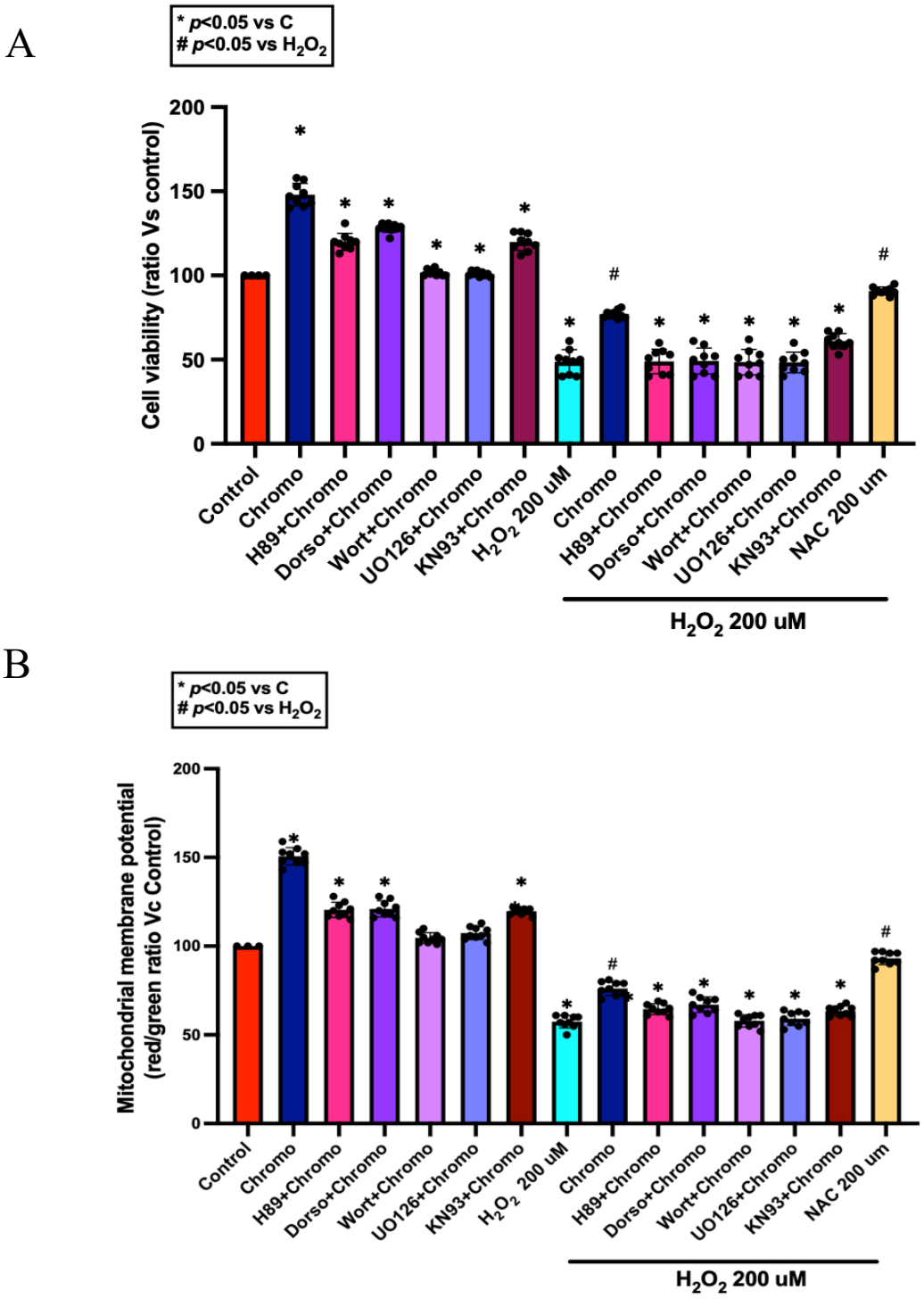



**Figure 13**: Impact of 10 nM chromogranin B on cell viability (Panel A) and mitochondrial membrane potential (Panel B) in HUVECs, assessed in the presence or absence of specific pathway modulators. Data represent medians with ranges from three independent experiments, each performed on separate HUVEC pools with triplicate measurements.
Dorso = dorsomorphin (AMPK inhibitor, 10 nM); H89 = PKA inhibitor (10 nM); KN93 = CaMKII inhibitor (10 nM); UO126 = MEK1/2 inhibitor (10 nM); Wort = wortmannin (broad PI3K inhibitor, 10 nM). Other abbreviations follow those in earlier figures.
In both panels (A and B), the Mann–Whitney U test was applied to compare the effects of chromogranin B in combination with each inhibitor to both the control group and the $H_2O_2$-treated group ("*" and "#" indicate these comparisons, respectively). A p-value $< 0.05$ was considered statistically significant (Grossini E, 2024).

As for NO release, the enhancement seen after 30 minutes of stimulation with 10 nM CgB under physiological conditions was further increased by isoproterenol, but abolished by the $\beta_2$-adrenergic antagonist butoxamine, and the inhibitors dorsomorphin, wortmannin, U0126, H89, KN93 (all at 10 nM), and L-NAME (10 mM) (Fig. 14).

In contrast, the muscarinic receptor antagonist atropine, the α-adrenergic receptor agonist phenylephrine, and the α-adrenergic receptor antagonist phentolamine did not influence the response of HUVECs to CgB (Fig. 14A). In HUVECs exposed to $H_2O_2$, the beneficial actions of CgB were also diminished by treatment with butoxamine, dorsomorphin, wortmannin, U0126, H89, and KN93 (each at 10 nM; Fig. 14) (Grossini E, 2024).

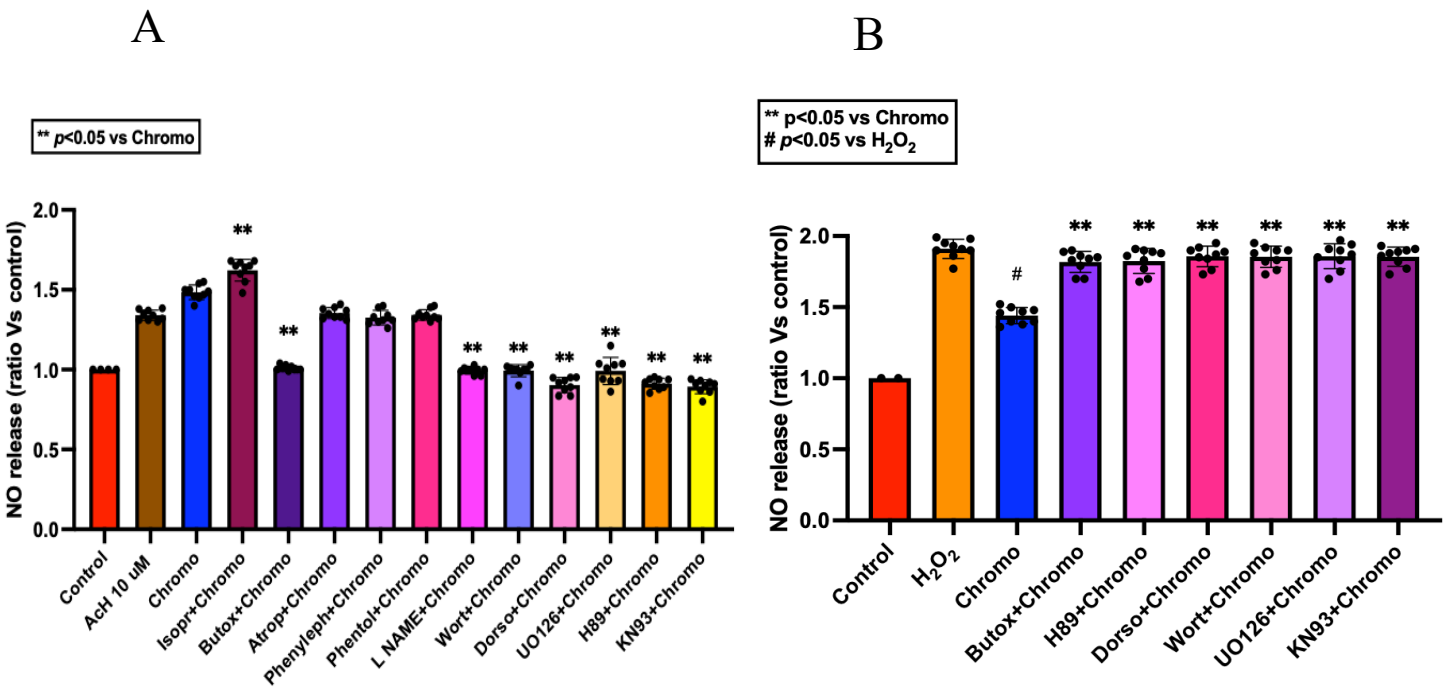


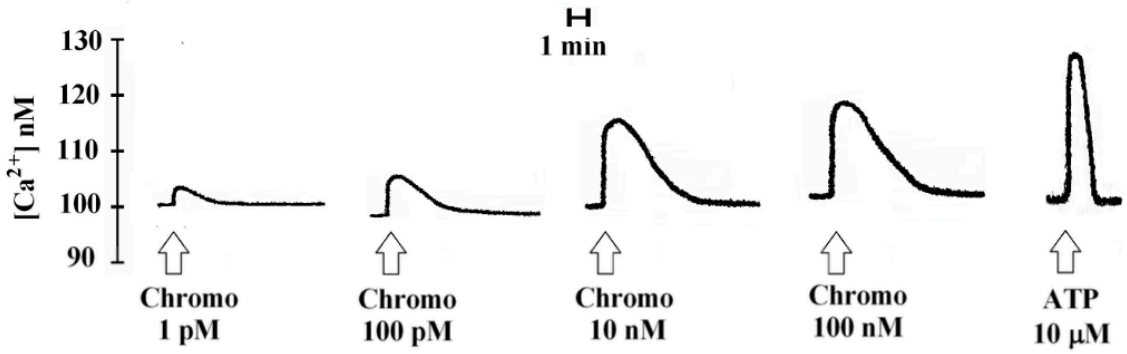

**Figure 14:** Impact of 10 nM chromogranin B on nitric oxide (NO) production in HUVECs, assessed in the presence or absence of various pharmacological agents. The data represent median values and range from experiments repeated three times on independent HUVEC pools, each measured in triplicate.

Atrop = atropine (cholinergic receptor blocker; 10 nM), Butox = butoxamine ($\beta_2$-adrenergic receptor antagonist; 10 nM), Isopr = isoproterenol (β-adrenergic receptor agonist; 10 nM), L-NAME = Nω-Nitro-L-arginine methyl ester hydrochloride (inhibitor of NO synthesis; 10 mM), Phenyleph = phenylephrine (α-adrenergic receptor agonist; 10 nM), Phentol = phentolamine (α-adrenergic receptor antagonist; 10 nM). All other abbreviations are consistent with previous figures.

In panels (A, B), statistical analysis was performed using the Mann–Whitney test to compare the effects of chromogranin B in combination with different agonists or antagonists (symbols "**" and "#" were analyzed together) to both control and $H_2O_2$-treated samples. A p-value $< 0.05$ was considered statistically significant (Grossini E, 2024).

As illustrated in Fig. 15, CgB triggered a dose-dependent and short-lived elevation in intracellular calcium concentration ([$Ca^{2+}$]c) in HUVECs maintained under normal conditions. The most pronounced increase in [$Ca^{2+}$]c was seen at 10 nM CgB. This rise in intracellular calcium peaked approximately one minute after stimulation with CgB and returned to baseline levels within about three minutes (Fig. 15) (Grossini E, 2024).

A




B

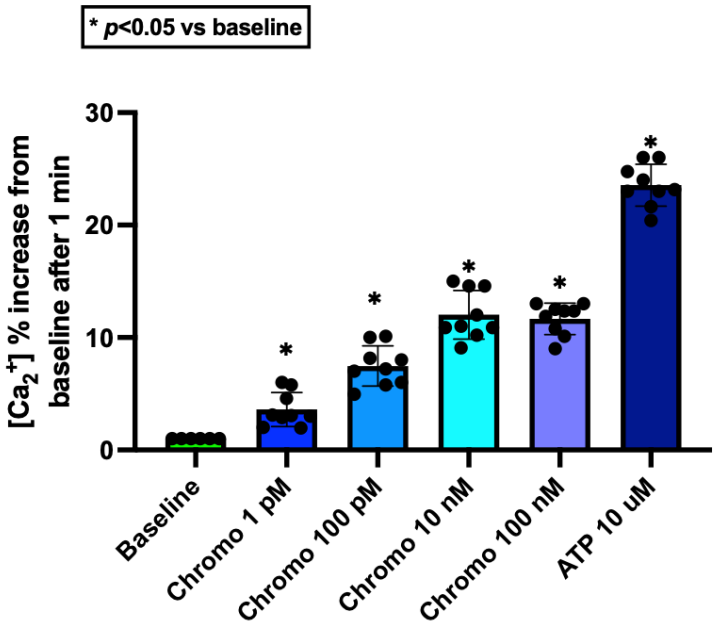



**Figure 15**: Effects of chromogranin B (1 pM, 100 pM, 10 nM, and 100 nM) on intracellular calcium concentration ([$Ca^{2+}$]c) in HUVECs cultured under physiological conditions. Panel (A) shows representative calcium traces. Panel (B) reports quantitative data collected 1 minute after stimulation. Results derive from three independent experiments conducted on different HUVEC pools, each performed with triplicate measurements, and are presented as medians with ranges. Abbreviations are consistent with those used in previous figures. In panel (B), statistical comparisons between chromogranin B concentrations and baseline values were performed using the Mann–Whitney test (asterisks were considered collectively). Statistical significance was set at $p < 0.05$ (Grossini E, 2024).

As illustrated in Fig. 16, the effects of 10 nM CgB on [$Ca^{2+}$]c were nearly eliminated in HUVECs maintained in a calcium-free solution (50 mM EGTA). Furthermore, the effect was enhanced by isoproterenol but completely blocked by butoxamine, H89, and KN93 (each at 10 nM) (Grossini E, 2024).

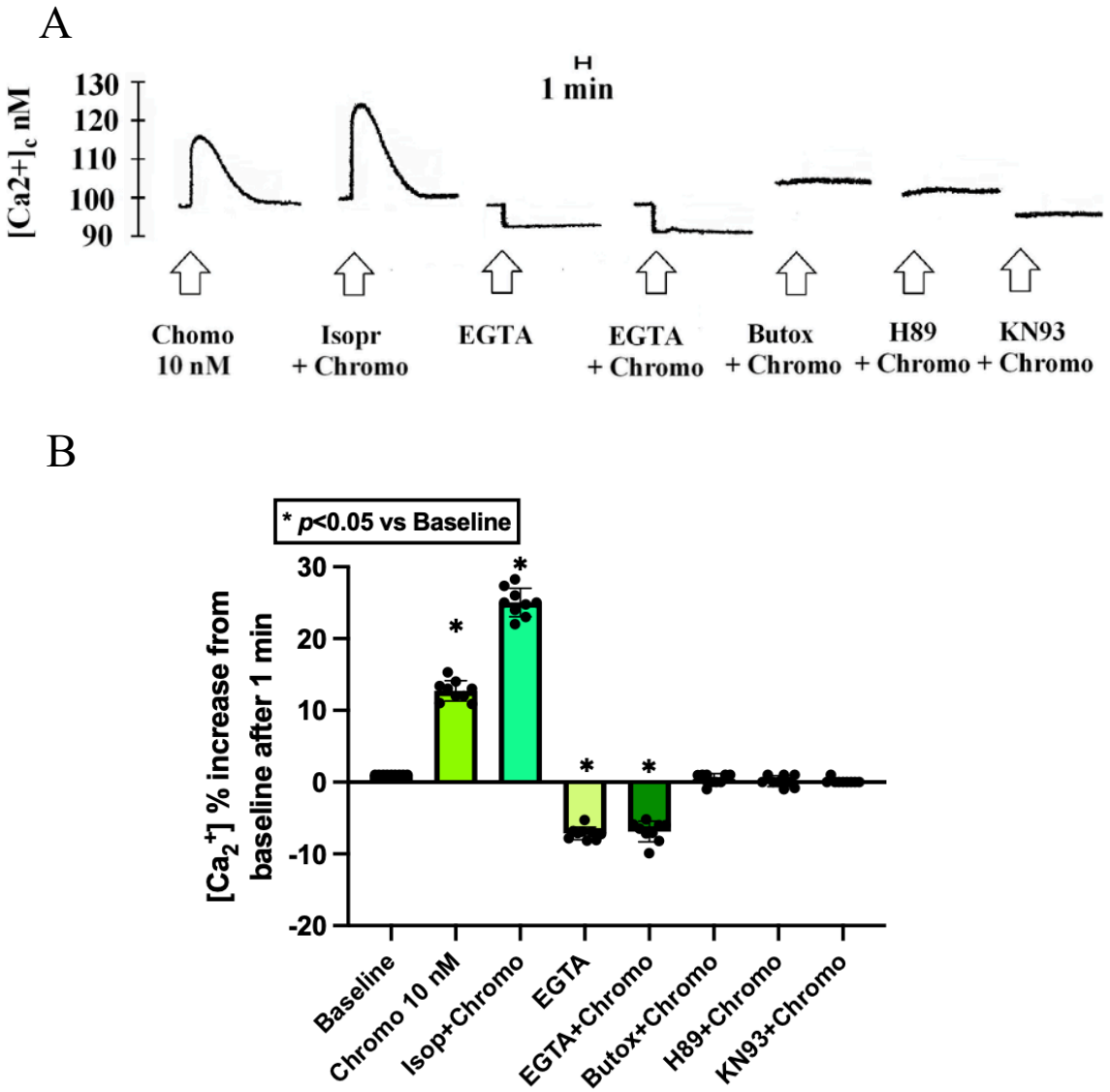



**Figure 16**: Effects of 10 nM chromogranin B on intracellular calcium concentration ([$Ca^{2+}$]c) in HUVECs cultured under physiological conditions, in the presence or absence of specific agonists/antagonists and ethylene glycol tetraacetic acid (EGTA). Panel (A) shows representative calcium traces. Panel (B) reports quantitative data collected 1 minute after stimulation.
Results derive from three independent experiments conducted on different HUVEC pools, each performed with triplicate measurements, and are presented as medians with ranges. Abbreviations are consistent with those used in previous figures: Chromo, chromogranin; Isopr, isoproterenol (10 nM); EGTA, ethylene glycol tetraacetic acid (50 mM); Butox, butoxamine (10 nM); H89, PKA inhibitor (10 nM); KN93, CaMKII inhibitor (10 nM).
In panel (B), statistical comparisons between chromogranin B in the presence of the various agonists/antagonists or EGTA and baseline values were performed using the Mann–Whitney test (asterisks were considered collectively). Statistical significance was set at $p < 0.05$ (Grossini E, 2024).

CgB demonstrated a dose-dependent ability to mitigate the impact of $H_2O_2$ on intracellular calcium levels ($[Ca^{2+}]c$) in HUVECs. As illustrated in Fig. 17, although $H_2O_2$ continued to elevate $[Ca^{2+}]c$, in the presence of CgB the increase was less pronounced and the sustained phase of the response was diminished. Notably, the most significant effects were observed in cells exposed to 10 nM of CgB (Grossini E, 2024).

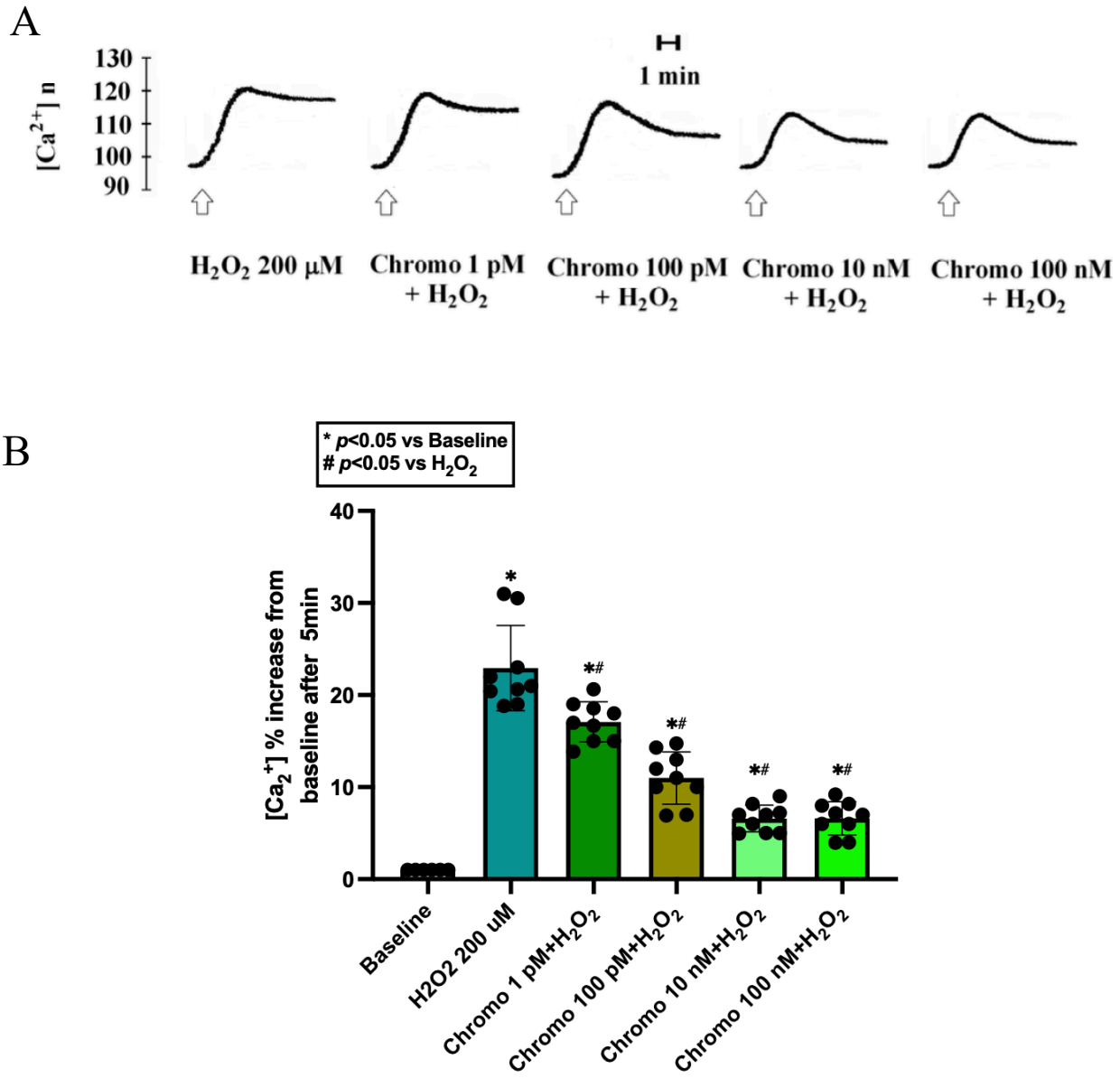



**Figure 17**: Effects of chromogranin B (1 pM, 100 pM, 10 nM, and 100 nM) on intracellular calcium concentration ($[Ca^{2+}]c$) in HUVECs cultured under peroxidative conditions. Panel (A) shows representative calcium traces. Panel (B) reports quantitative data collected 5 minutes after stimulation. Results derive from three independent experiments conducted on different HUVEC pools, each performed with triplicate measurements, and are presented as medians with ranges. Abbreviations are consistent with those used in previous figures.
In panel (B), statistical comparisons between the different chromogranin B concentrations and baseline as well as $H_2O_2$ were performed using the Mann–Whitney test (asterisks and hash symbols were considered collectively, respectively). Statistical significance was set at $p < 0.05$ (Grossini E, 2024).

# 5. DISCUSSION

This study demonstrated that CgB enhances cell survival, NO production and mitochondrial activity in HUVECs while mitigating oxidative damage. Specifically, we observed that CgB significantly decreased the impact of hydrogen peroxide ($H_2O_2$) on calcium signaling, NO and ROS levels, and helped preserve GSH levels. Under physiological conditions, CgB rapidly and transiently elevated intracellular calcium levels ($[Ca^{2+}]_c$), but under oxidative stress, it reduced the calcium increase typically caused by $H_2O_2$. These effects were associated with multiple signaling pathways, including those involving β-adrenergic receptors, PKA, AMPK, MEK1/2-ERK1/2, PI3K/Akt, and CaMKII. While the actions of CgA and its derivatives have been well documented in a variety of cardiovascular cell types (e.g., endothelial cells, cardiomyocytes, vascular smooth muscle), data on CgB remain scarce (Grossini E, 2024).

CgA-derived peptides such as vasostatin I and II and catestatin have shown a range of vascular effects, including promoting or inhibiting vasodilation, cell proliferation, migration, and angiogenesis, through mechanisms involving potassium channels and signaling pathways such as ERK and PI3K (Taupenot, 2003) (Louthan, 2011).

In contrast, CgB is primarily known for its role in the formation and secretion of catecholamine vesicles and its influence on autonomic regulation of blood pressure. Furthermore, elevated plasma levels of CgB have been identified as an independent predictor of ventricular function recovery post-cardiac intervention. However, direct evidence of CgB's effects on endothelial function has been lacking. Since the cardiovascular effects of CgB may extend beyond its role in catecholamine release, we sought to explore its direct influence on endothelial physiology (Troger, 2017).

To investigate this, we evaluated CgB's effects in HUVECs exposed to both physiological and oxidative conditions, focusing on key indicators such as viability, mitochondrial function, antioxidant defense, and NO output. An initial dose- and time-response study established that CgB concentrations ranging from 1 pM to 10 nM enhanced cell viability and mitochondrial membrane potential, with peak effects at 10 nM. NO release also increased in a similar concentration and time-dependent fashion. It is to note that those concentrations were similar to those found in the plasma of normal subjects and patients with respiratory failure, heart failure, angina, and neuroendocrine tumors (Ramachandran, 2015) (Monaghan, 2016) (Stridsberg, 1997) (Røsjø H. D., 2010).

As regarding the time-course, CgB-induced effects on cell viability and NO release were time-dependent, showing a progressive increase that reached its peak at 30 minutes (Grossini E, 2024).

From the results obtained, we selected the 10 nM concentration and 30 minute timing to perform the other experiments.

Under oxidative conditions induced by $H_2O_2$, CgB improved cell viability and mitochondrial function while reducing NO and ROS production. These protective effects were accompanied by higher GSH levels. The ability of CgB to mitigate mitochondrial dysfunction is particularly significant, given the central role of mitochondrial damage in disrupting endothelial function and promoting hypertension. Changes in mitochondrial membrane potential can lead to excess ROS generation, activating inflammatory pathways and decreasing NO availability, thus shifting the endothelial balance towards vasoconstriction and vascular disease (Qu, 2022).

Our findings point to a dual role for CgB in modulating NO release: enhancing it under normal conditions and suppressing it under oxidative stress. This behavior mirrors responses seen with other agents (e.g., hCG, asenapine, artemetin) and highlights the complexity of NO regulation, which is affected by oxidative stress and differential activation of endothelial (eNOS) versus inducible nitric oxide synthase (iNOS). CgB's effects were shown to involve a variety of intracellular pathways, notably PKA, AMPK, PI3K/Akt, MEK1/2-ERK1/2, and CaMKII. These pathways are crucial not only for regulating NO production via eNOS but also for supporting cell survival (Zhang, 2009) (Wu, 2021).

The enhancement of NO release by isoproterenol and its blockade by the β2-adrenergic receptor antagonist butoxamine confirm the receptor-specific actions of CgB (Grossini E, 2024) (Sandoo, 2010) (Rochette, 2013).

Regarding calcium dynamics, CgB elicited a dose-dependent increase in $[Ca^{2+}]c$ under physiological conditions and decreased $H_2O_2$-induced calcium elevation under oxidative stress. The attenuation of CgB's calcium-mobilizing effects by EGTA confirmed the extracellular origin of calcium influx. Moreover, pharmacological interventions using H89, KN93, isoproterenol, and butoxamine confirmed the role of PKA, CaMKII, and β2-adrenergic receptors in mediating these effects.

Overall, these results provide important insights into the endothelial actions of CgB, revealing parallels with other chromogranin peptides, particularly regarding signaling via PI3K/Akt, MEK1/2, and β-adrenergic receptors.

These findings suggest that under normal conditions, CgB activates eNOS via multiple pathways, including increases in $[Ca^{2+}]c$, while under oxidative stress, it may downregulate both eNOS and iNOS activity, contributing to reduced NO and ROS levels. This dual regulation may underlie CgB's mitochondrial protection and beneficial effects on endothelium (Grossini E, 2024).

These findings are particularly relevant considering the central role of mitochondria, redox state balance, and NO in maintaining vascular endothelial function. Endothelial dysfunction, a common early event in vascular diseases, can arise from mitochondrial damage [24]. In endothelial cells, unbalanced ROS generation, not countered by ROS-scavenging antioxidant systems, is considered a major risk factor for the development and progression of atherosclerosis [40]. Increased ROS production can reduce NO bioavailability, leading to arterial vasoconstriction and contributing to the development of hypertension (Panda, 2022) (Griendling, 2021).

By this way, our data provide new information about the direct role of CgB in the physiological regulation of vascular endothelial cells function and may hold clinical relevance. Hence, by modulating endothelial function and calcium signaling, CgB could exert protective effects in conditions characterized by oxidative stress, such as heart failure, coronary artery disease, and hypertension. This supports prior observations of an inverse relationship between CgB levels and blood pressure, suggesting a potential therapeutic role for CgB in cardiovascular disease (Grossini E, 2024).

Anyway, further studies using techniques such as immunofluorescence, Western blotting and cGMP quantification, along with β2-adrenergic blockade, are necessary to elucidate the detailed mechanisms involved. Investigating CgB's impact on endothelial cell proliferation, migration, and interaction with catecholamines may provide deeper understanding of its may provide a deeper understanding of its role in endothelial physiology and vascular homeostasis.

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