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ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΤΟΜΕΑΣ ΠΑΘΟΛΟΓΙΑΣ
Α' ΚΑΡΔΙΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

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ΕΛΕΝΗ – ΤΑΞΙΑΡΧΙΑ ΜΟΥΧΤΟΥΡΗ

ΒΙΟΛΟΓΟΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΙΩΑΝΝΙΝΑ 2023

ΠΑΝΕΠΙΣΤΗΜΙΟ ΙΩΑΝΝΙΝΩΝ
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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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Η υλοποίηση της διδακτορικής διατριβής συγχρηματοδοτήθηκε από την Ελλάδα και την Ευρωπαϊκή Ένωση (Ευρωπαϊκό Κοινωνικό Ταμείο) μέσω του Επιχειρησιακού Προγράμματος «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Δια Βίου Μάθηση», 2014-2020, στο πλαίσιο της Πράξης «Ενίσχυση του ανθρώπινου δυναμικού μέσω της υλοποίησης διδακτορικής έρευνας Υποδράση 2: Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ σε υποψηφίους διδάκτορες των ΑΕΙ της Ελλάδας.

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Εκπαίδευση και Διά Βίου Μάθηση
Με τη συγχρηματοδότηση της Ελλάδας και της Ευρωπαϊκής Ένωσης

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Ιωάννινα 03-11-2023
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Η Γραμματέας του Τμήματος

ΜΑΡΙΑ ΚΑΠΙΤΟΠΟΥΛΟΥ

Αφιερωμένο στην οικογένεια και στους φίλους μου.

ΠΡΟΛΟΓΟΣ

Η καρδιά είναι ένα μοναδικό και ζωτικής σημασίας όργανο, που καθορίζει τη βιωσιμότητα όλων των υπόλοιπων οργάνων του ανθρώπινου σώματος. Η πολυπλοκότητα της λειτουργίας της είναι και το χαρακτηριστικό που την καθιστά τόσο μοναδική. Δυστυχώς, τα νοσήματα που διαταράσσουν τη λειτουργία της καρδιάς, με κύριο πρωταγωνιστή τη Στεφανιαία Νόσο, είναι υπεύθυνα για το θάνατο χιλιάδων ανθρώπων. Ένας από τους βασικούς λόγους της αύξησης του ποσοστού των θανάτων που οφείλονται σε καρδιαγγειακά νοσήματα και παρατηρείται τις τελευταίες δεκαετίες, είναι και ο σύγχρονος τρόπος ζωής. Ανεξάρτητα από το πως οι άνθρωποι εκτίθενται σε οξύ συναισθηματικό στρες, είναι πιθανό μετά από αυτό να ακολουθήσει αυξημένος κίνδυνος οξείας στεφανιαίας νόσου και αύξηση της συμπαθητικής δραστηριότητας συνοδευόμενη από έντονη καρδιαγγειακή απόκριση. Ο αιφνίδιος καρδιακός θάνατος, που συχνά προκαλείται από κοιλιακές ταχυαρρυθμίες κατά την αρχική φάση της οξείας στεφανιαίας απόφραξης, αντιπροσωπεύει το 13% της θνησιμότητας από φυσικά αίτια στο γενικό πληθυσμό και έχει αναδειχθεί ως ένα πολύ σημαντικό πρόβλημα. Πρόσφατες μελέτες εμπλέκουν την ενδοθηλίνη, ένα πανταχού παρόν πεπτίδιο, ως σημαντικό ρυθμιστή οξείας και χρόνιας συμπαθητικής ενεργοποίησης. Εκτός από άμεσες αρρυθμιογόνες δράσεις, η ενδοθηλίνη μπορεί να εμπλέκεται και στη ρύθμιση του συμπαθητικού συστήματος όσον αφορά στην ηλεκτροφυσιολογία της καρδιάς και κατά συνέπεια, μπορεί να διαδραματίζει ρόλο στην παθογένεση των διαταραχών του καρδιακού ρυθμού.

Η παρούσα διατριβή θα εξετάσει τα αποτελέσματα της κεντρικής συμπαθητικής ενεργοποίησης στην ηλεκτροφυσιολογία της καρδιάς και στην αρρυθμιογένεση που προκαλείται από ισχαιμία του μυοκαρδίου. Επιπλέον, θα εξεταστεί ο ρόλος της ενδοθηλίνης στις συμπαθητικές αποκρίσεις στην οξεία ισχαιμία του μυοκαρδίου και στη ρύθμιση του οξέος συναισθηματικού στρες, με σκοπό τη συλλογή γνώσεων που ενδεχομένως αποτελέσουν βάση για τη δημιουργία νέων θεραπευτικών προσεγγίσεων στους ασθενείς με ισχαιμία του μυοκαρδίου που εμφανίζουν ταχυαρρυθμίες και οδηγούνται σε αιφνίδιο καρδιακό θάνατο. Το πρώτο και απαραίτητο βήμα

ώστε να υλοποιηθεί μια ιδέα και πιθανόν να εφαρμοστεί στο μέλλον στην κλινική πράξη, είναι η πειραματική μελέτη. Η συγκεκριμένη μελέτη πραγματοποιήθηκε σε πειραματικό ζωικό πρότυπο σύμφωνα με τις κατευθυντήριες οδηγίες των 3Rs. Η παρουσίαση αυτής της μελέτης γίνεται σε τρία ξεχωριστά μέρη. Στο πρώτο, το γενικό μέρος, γίνεται βιβλιογραφική ανασκόπηση και παρουσιάζεται η θεωρητική γνώση που οδήγησε στη σύλληψη της ανωτέρω ιδέας. Στο δεύτερο, περιγράφεται ο σκοπός της συγκεκριμένης διδακτορικής διατριβής. Στο τρίτο, το ειδικό μέρος, παρουσιάζονται λεπτομερώς ο σχεδιασμός του πειραματικού πρωτοκόλλου, τα υλικά και οι μέθοδοι που χρησιμοποιήθηκαν για να υλοποιηθεί το συγκεκριμένο πειραματικό πρωτόκολλο, καθώς και τα αποτελέσματα που προέκυψαν από την ολοκλήρωσή του. Στο τέταρτο και τελευταίο μέρος, περιλαμβάνονται συνοπτικά τα συμπεράσματα στα οποία οδηγήθηκα από την ολοκλήρωση της διατριβής.

Στο σημείο αυτό, θα ήθελα να εκφράσω τις ευχαριστίες μου σε όλους τους ανθρώπους που με στήριξαν και στάθηκαν δίπλα μου σε όλη αυτή την προσπάθεια. Αρχικά, τις πιο θερμές μου ευχαριστίες και τη βαθύτατη ευγνωμοσύνη μου οφείλω στον Καθηγητή Καρδιολογίας και επιβλέποντα μου, κύριο Θεόφιλο Κωλέττη, που με δέχτηκε στην ερευνητική του ομάδα. Θα ήθελα να τον ευχαριστήσω, ιδιαίτερα, για τη συνεχή καθοδήγησή του, την πολύτιμη στήριξή του, αλλά κυρίως επειδή μου μετέδωσε τον άρτιο και επιστημονικό τρόπο σκέψης του αλλά και το πνεύμα της συνεχούς επιστημονικής αναζήτησης που τον διακρίνει. Εκφράζω την ευγνωμοσύνη μου, διότι, με τις πολύτιμες συμβουλές και τις ερευνητικές κατευθύνσεις του, στάθηκε δίπλα μου, σε κάθε βήμα, όλα αυτά τα χρόνια και κατέστησε δυνατή την ολοκλήρωση αυτής της διατριβής.

Ένα πολύ μεγάλο ευχαριστώ οφείλω στον Καθηγητή Φαρμακολογίας, κύριο Κωνσταντίνο Πάντο και στον Αναπληρωτή Καθηγητή Φαρμακολογίας, κύριο Ιορδάνη Μουρούζη για την ουσιαστική βοήθεια που μου προσέφεραν αλλά και για τη φιλοξενία τους στις εγκαταστάσεις και στους χώρους του εργαστηρίου Φαρμακολογίας της Ιατρικής Σχολής Αθηνών. Ήταν πάντα πρόθυμοι να απαντήσουν σε κάθε απορία μου και συνέβαλλαν σημαντικά στην εξεύρεση λύσεων, στις όποιες δυσκολίες παρουσιαζόταν, κατά τη διάρκεια όλων αυτώ των ετών.

Θα ήταν παράληψή μου, αν δεν εξέφραζα την πιο βαθιά μου ευγνωμοσύνη σε όλους τους συναδέλφους και συνεργάτες του εργαστηρίου Ηλεκτροφυσιολογίας της Καρδιάς, με τη βοήθεια των οποίων έγινε δυνατή η ολοκλήρωση της παρούσας διατριβής. Ειδικότερα στους πολύτιμους συνεργάτες μου όλα αυτά τα χρόνια Παναγιώτη Λέκκα και Θωμά Κωνσταντίνου, τόσο για την έμπρακτη βοήθειά τους στην υλοποίηση του μεγαλύτερου μέρους της πειραματικής διαδικασίας του πρωτοκόλλου αλλά και γιατί ήταν δίπλα μου κάθε στιγμή και με στήριζαν τόσο σε εργαστηριακό αλλά και σε προσωπικό επίπεδο. Επιπλέον, θα ήθελα να εκφράσω τις ευχαριστίες μου και στην Αλεξάνδρα Λιανοπούλου και στη Ζωή Κοτσαρίδου για τη συμβολή τους στην επεξεργασία των αποτελεσμάτων της διατριβής αλλά και στην Ελένη Γκόγκα για την πολύτιμη καθοδήγησή της στο διαδικαστικό κομμάτι της διδακτορικής μου διατριβής. Ακόμη, ένα μεγάλο ευχαριστώ στη Valbona Bicaku για την κατασκευή του ειδικού restrainer για την πρόκληση στρες στους επίμυες.

Τέλος, ευχαριστώ την οικογένεια μου για τη στήριξή τους καθ' όλη τη διάρκεια εκπόνησης της διδακτορικής μου διατριβής. Ιδιαίτερα θέλω να ευχαριστήσω τη μητέρα μου, Γιώτα, καθώς αποτέλεσε τόσο τον λόγο για να ξεκινήσω όσο και την κινητήρια δύναμη για να ολοκληρώσω τη διατριβή μου.

Ελένη – Ταξιαρχία Μουχτούρη

Ιωάννινα 2023

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ABBREVIATIONS

ANS: Autonomic nervous system

AES: Acute emotional stress

AJS: Air jet stress

AV: Atrio – ventricular

BP: Blood pressure

BPM: Beats per minute

CAD: Coronary artery disease

ECE: Endothelin converting enzyme

ECG: Electrocardiogram

ET-1: Endothelin-1

ET-2: Endothelin-2

ET-3: Endothelin-3

ETA: Endothelin receptor A

ETB: Endothelin receptor B

HF: Heart failure

HF: High frequency

HPA: Hypothalamic-pituitary-adrenal

HR: Heart rate

HRV: Heart rate variability

LF: Low frequency

LV: Left ventricle

LVEF: Left ventricular ejection fraction

MAP: Mean arterial pressure

MI: Myocardial Infarction

NO: Nitric oxide

NYHA: New York Heart Association

PBS: Phosphate buffered saline

PNS: Parasympathetic nervous system

PNSi: Parasympathetic nervous system index

PVC: Premature ventricular contraction

RMSSD: root mean square of successive differences between inter-beat intervals in time-domain analysis

SDNN: Standard deviation of RR intervals

SNS: Sympathetic nervous system

SNSi: Sympathetic nervous system index

VF: Ventricular fibrillation

VT: Ventricular tachycardia

A. GENERAL INTRODUCTION

1. ENDOTHELIN PHYSIOLOGY

1.1 GENERAL INFORMATION

The discovery of endothelin (now called endothelin-1 or ET-1), an endothelium-derived constricting factor, by Yanagishawa in 1988 was a milestone in the field of cardiovascular research **(1)**. Pharmacological and molecular approaches revealed that endothelin was the most potent vasoconstrictor ever described to date, producing extremely powerful and long lasting contraction of a range of mammalian blood vessels in vitro, including human arteries and veins. Later, in humans, two further peptides (Fig.1), endothelin-2 (ET-2) and endothelin-3 (ET-3), were identified to complete the family of endogenous endothelin agonists **(2)**.

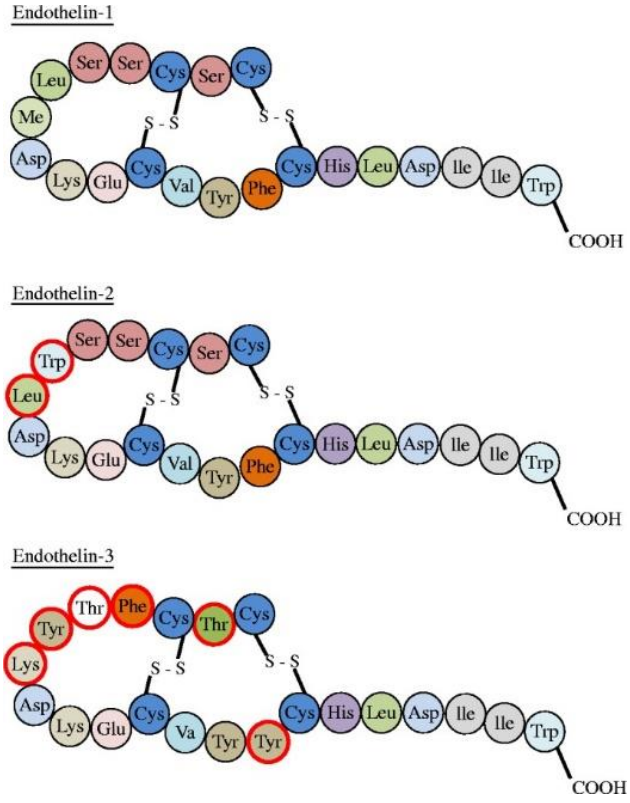


Figure 1: *Amino-acid structure of endothelin isoforms. Changes in specific amino acids in the peptide sequence compared to ET-1 are circled in red. (From reference **(3)**. With permission.)*

Since their discovery, many efforts have been made to understand the role played by these peptides, physiologically and pathophysiologically, particularly in relation to the cardiovascular system.

1.2 ENDOTHELIN IN A MOLECULAR LEVEL

As mentioned before, endothelin is expressed through three isoforms: ET-1, ET-2, and ET-3. All three forms are peptides consisting of 21 amino acids with a free amino terminus and C-terminal carboxylic acid, but expressed by separate genes **(1, 4)**. Endothelins are secreted by a variety of cell types and function in both autocrine and paracrine signalling **(5, 6)**. In humans, ET-1 is the major isoform synthesized mainly in the cardiovascular system, and the one studied the most. The primary source of ET-1 in the cardiovascular system is thought to be vascular endothelial cells. It is, also, synthesized and secreted by other cell types, including epithelial cells, for example in the kidney, the lungs and the colon. In the periphery, it is additionally produced in macrophages and monocytes, enteric glia cells, as well as choroid plexus and certain neurons and reactive glial cells in the central nervous system. ET-2 is mainly synthesized in the kidneys and the gastrointestinal system, while ET-3 is mainly found in the central nervous system leading to its characterization as the "brain" endothelin peptide **(6)**.

Table 1. *ET-1, ET-2 and ET-3 synthesis sites.*

ET-1	ET-2	ET-3
Endothelial cells	Epithelial kidney cells	Neurons
Vascular smooth muscle cells	Gastrointestinal stromal cells	Glial cells
Epithelial cells		Epithelial lung cells
Hepatocytes		Gastrointestinal stromal cells
Neurons		Epithelial kidney cells
Astrocytes		Purkinje cells

1.3 ENDOTHELIN-1 SYNTHESIS

ET-1 is produced after exposure to a wide range of stimuli, which varies between different tissues **(4)**. This peptide is mainly synthesized in the nucleus of vascular endothelial cells but follows a specific pathway (Fig. 2), before reaching its final form. The release of active ET-1 is controlled via regulation of its gene transcription and/or the endothelin converting enzyme activity.

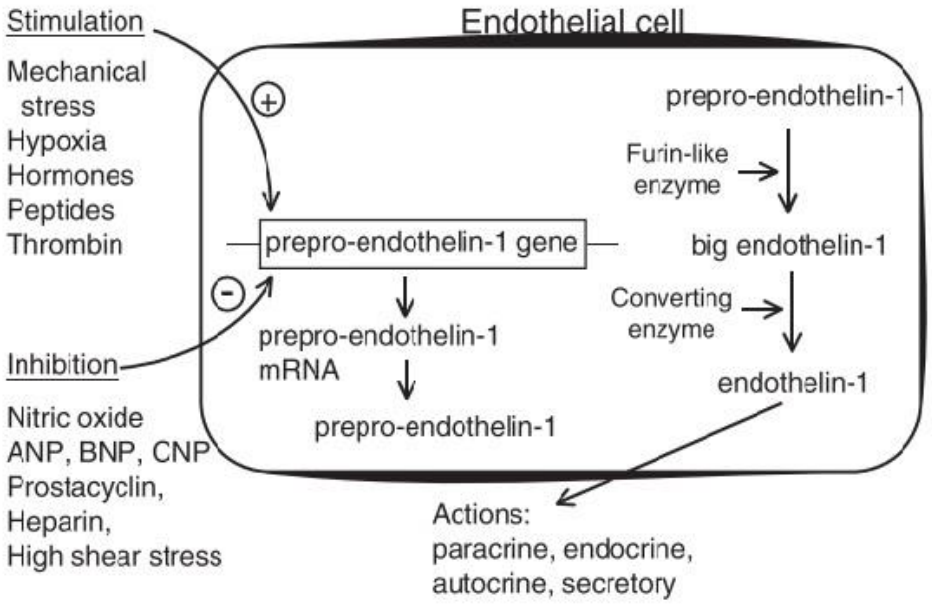


Figure 2: *Synthesis of ET-1 and its regulation. Afferent stimuli promote or inhibit ET-1 synthesis. For example, angiotensin II, vasopressin, cytokines, thrombin, oxygen free radicals and wall stress are factors that enhance ET-1 production. In contrast, NO, prostacyclin and atrial natriuretic peptide (ANP) are substances that reduce ET-1 synthesis. (Modified from reference **(7)**. With permission)*

As can be seen in figure 2, there are hormones, peptides, blood components, physical and chemical factors, as well as other factors that contribute positively to the ET-1 gene transcription, which leads to an increase ET-1 synthesis. Some of these stimulating factors are adrenaline, angiotensin II, vasopressin, stress hormones in general, hypoxia, wall stress, thrombin, the

oxidized form of LDL cholesterol, etc. The stimulatory effects involve cellular Ca^{2+} mobilization, in general. By contrast, other factors, such as prostacyclin, NO, heparin, high shear stress, atrial natriuretic peptide, etc. inhibit ET-1 gene transcription **(6, 7)**. ET-1 can be secreted rapidly, in response to stimuli of short duration, implying that this peptide is partially stored in endothelial cells rather than being synthesized de novo **(8)**.

In summary, ET-1 synthesis includes the following stages:

1) Stimulation of the ET-1 gene, located in the nucleus of the endothelial cell.
2) Generation of prepro-ET-1 mRNA, a precursor form of ET-1.
3) Synthesis of prepro-ET-1 in the cytoplasm of the cell.
4) Catalysis of prepro-ET-1 to big endothelin-1 by furin-like enzyme.
5) Cleavage of big endothelin-1, an aminopeptide of 38 amino acids, into ET-1 by ET-1 converting enzyme (ECE-1), a hyper selective metalloproteinase.

ET-1 acts as a paracrine molecule and possibly as an endocrine as well. That is why classification between big ET-1 and ET-1 needs special attention. The factors that regulate the clearance and less the synthesis of the molecule are among the most important indicators of its concentration in the plasma.

Although the biological effects of ET-1 last much longer, its half-life is less than two minutes. This is due to its excretion from the pulmonary and the renal vascular system. This excretion involves the binding of ET-1 by B-receptors located on the cell surface, its absorption and its degradation in the lysosomes and it was first described by Fukuroda et al. in 1994 **(9)**. The important role of ETB receptors in clearing ET-1 from the circulation, is now well established and may primarily serve to keep tissue ET-1 concentrations low, but it can also be used to advance animal experimental models with high circulating levels of ET-1 **(7, 10)**. Endothelin molecules are also degraded by endopeptidases located in the proximal convoluted tubule in the kidney.

1.4 ENDOTHELIN RECEPTORS

In mammalian tissues the three endothelin isoforms exert their actions by binding to two specific receptors, receptor-A (ETA) and receptor-B (ETB) **(11)**. Both of them are G protein-coupled, 7 transmembrane-spanning domain receptors (GPCR) and they are located in the cell membrane. However, the human ETA receptor has 63% similarity in its sequence with the human ETB receptor, over a 420-residue match length. The existence of further ET receptor subtypes is suggested by published studies: for instance, ETB isoforms were suggested with ETB1 present on endothelial cells and ETB2 on smooth muscle cells, but the evidence is against this theory **(12)**. The two main ET receptors are distributed in the human tissues as sown in Fig. 3, with the human brain containing a high density of ET receptors and the lungs having the highest density from peripheral tissues **(6)**. ET receptors have different molecular structures and distinct pharmacological properties and function based mainly on their location, since A receptors are mainly expressed on vascular smooth muscle cells, while B receptors on endothelial cells.

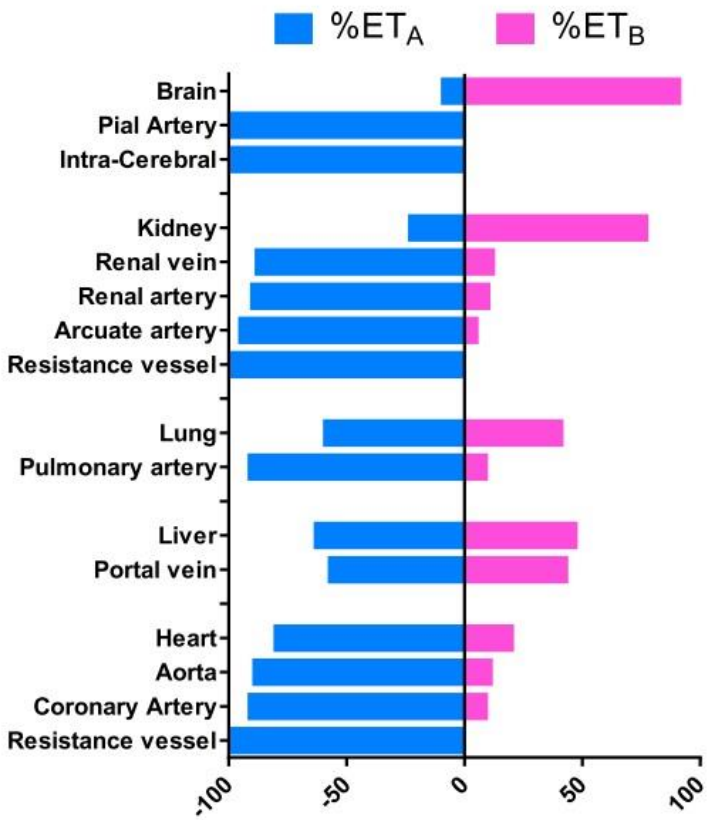


Figure 3: *Ratio of ETA to ETB densities in the human tissues. (From reference **(6)**. With permission)*

Table 2. *Properties of the two endothelin receptors.*

	ETA	***ETB***
Agonists affinity	*ET-1>ET-2>>ET-3*	*ET-1 = ET-2 = ET-3*
Agonists selectivity	*None*	*ET-3, sarafotoxin-6c, BQ3020, IRLI620*
Main actions	*Vasoconstriction*	*Vasodilation*
	Mitogenesis	*Vasodilation*
	Angiogenesis	*Endothelin excretion*

The binding of ET-1 to these receptors leads to the activation of the phospholipase C pathway and the triggering of intracellular processes (Fig.4), with short- and long-term effects. Such are the rapid increase in intracellular calcium levels, the activation of protein kinase C and nuclear signaling mechanisms. As mentioned before, ETA receptors are mainly expressed on vascular smooth muscle cells and myocardial cells. Their interaction with ET-1 leads to vasoconstriction, while they also stimulate cell growth. In contrast, ETB receptors are mainly found on vascular endothelial cells, where they are coupled to an inhibitory G protein. Their activation results in the release of NO and prostacyclin, which in turn lead to vasodilation. Receptors of B type are found, in much smaller concentrations, in smooth muscle cells of the vessels, where they contribute to vasoconstriction. Thus, in normal conditions, i.e. in the absence of diseases, the actions of ET-1 lead to maintenance of vascular tone, tissue differentiation and cell proliferation through the properties of ETA and ETB receptors. On the contrary, in pathological conditions, ET-1 receptors lead mainly to vasoconstriction and cell growth **(13-15)**.

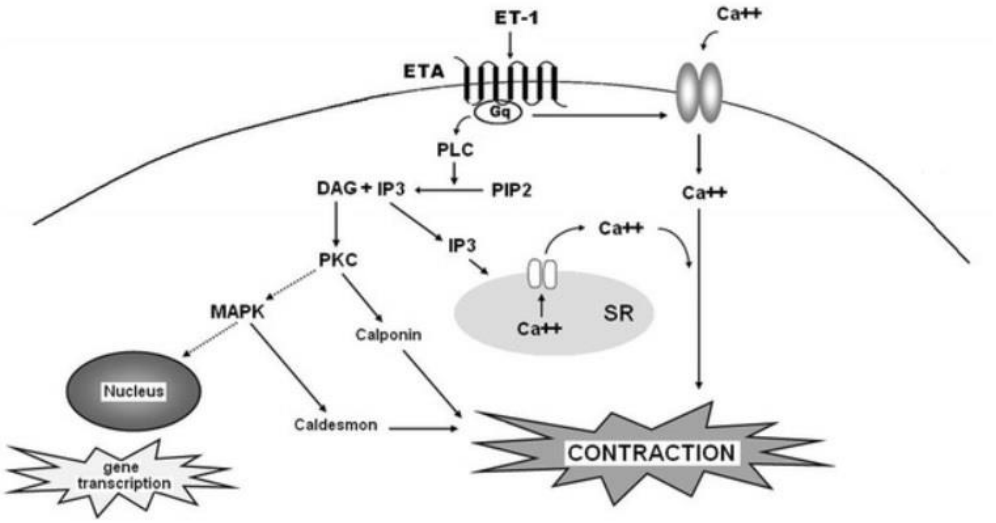


Figure 4: *ETA receptor downstream signalling pathways in the vascular smooth muscle cell. ETA receptor activation stimulates phospholipase (PLC) to produce IP3 and DAG. IP3 induces Ca^{2+} outflow from the sarcoplasmic reticulum (SR). Moreover, the ETA receptor acts on nonselective membrane Ca^{2+} channels causing Ca^{2+} input from the extracellular space. Consequently, increased concentrations of Ca^{2+} leads to the cell contraction. The activated ETA receptor also stimulates cell growth. Production of DAG activates protein kinase C (PKC), which is responsible for the mitogenic function of endothelin, and which also induces a Ca^{2+}-independent pathway of cell contraction involving calponin phosphorylation. PKC affects gene transcription through activation of the Ras/Raf/MEK/MAPK cascade. (Modified from reference* ***(16)****. With permission)*

1.5 ENDOTHELIN AGONISTS AND ANTAGONISTS

Understanding the role of ET-1 in human physiology comes not only from creating agonists, substances that facilitate its action, but also antagonists and ET-1 (directly labeled via Tyr13, a residue not critical for ligand-receptor interaction) that binds with the same affinity to both subtypes.

(a) ET-1 agonists

To date, no specific agonist for ETA receptors has been discovered. As for ETB receptors, sarafotoxin-6, found in snake venom, and ET-3, have been found to have a higher affinity for ETB than ETA receptor, thus they have been used as ETB receptors' agonists **(17)**. In humans, ET-1 induces a slow, dose-dependent vasoconstriction that persists for approximately two hours **(18).** This response is attenuated by infusing an ETA receptor antagonist **(19)**, indicating that ET-1-induced vasoconstriction is primarily mediated by ETA receptor. This action is followed by vasodilation possibly mediated by ETB receptors located on the vessels endothelial cells. In addition, intravenous injection of ET-1 and big ET-1 causes a dose-dependent decrease in blood pressure and heart rate **(20)**. These systemic effects are associated with a decrease in coronary blood flow and oxygen saturation in the heart atria. This fact points to the possible role of ET-1 in the maintenance of coronary vascular tone.

(b) ET-1 antagonists

Clinical and preclinical studies support the pathogenic role of ET-1 in a multitude of diseases. This fact pushed researchers in the direction of finding antagonists of ET-1 aiming to obtain possible positive results from their use at a clinical level. ET-1's antagonists are divided into two major categories: i) ET-1 converting enzyme inhibitors and ii) ET-1 receptor antagonists.

i) ET-1 converting enzyme inhibitors

Inhibition of ET-1 converting enzyme (ECE) prevents conversion of big ET-1 to ET-1 and is associated with vasodilation and hypotension. Most ECE inhibitors also inhibit endopeptidases, resulting in inhibit both ET-1 production and the metabolism of vasodilator mediators that are likely degraded by endopeptidases. Such mediators are atrial natriuretic peptide and bradykinin. Furthermore, the inhibition of angiotensin-converting enzyme (ACE) can also be added, with possible positive effects in pathological conditions such as heart failure and renal dysfunction. In addition, by inhibiting ET-1 synthesis, ECE inhibitors act as double antagonists, both for the ETA and ETB receptors, even by not affecting ET-1. Despite this, the progress achieved to date with ECE inhibitors is significantly poor compared to the ET-1 receptor antagonists **(21, 22)**.

ii) ET-1 receptor antagonists

ET-1 receptor antagonists are divided into selective ETAs or selective ETBs, depending on their affinity for the receptor type, or dual ETA/B receptor antagonists when they do not show any particular receptor affinity (Fig.5). The two widely used antagonists are BQ-123 (ETA receptor antagonist) and BQ-788 (ETB receptor antagonist). As for dual antagonists, a large number of substances have been used in experimental studies, but Bosentan, an orally administered non-peptide antagonist, was the first to receive approval for clinical use by the Food and Drug Administration (FDA), as well as by the European Medicines Agency (EMA) for the treatment of pulmonary hypertension **(23, 24)**. It should be noted that the distinction between selective and dual antagonists is not well defined. In particular, dual antagonists have a higher affinity for ETA receptors, while selective ETA receptors when used in relatively large doses can act on both receptors **(25)**.

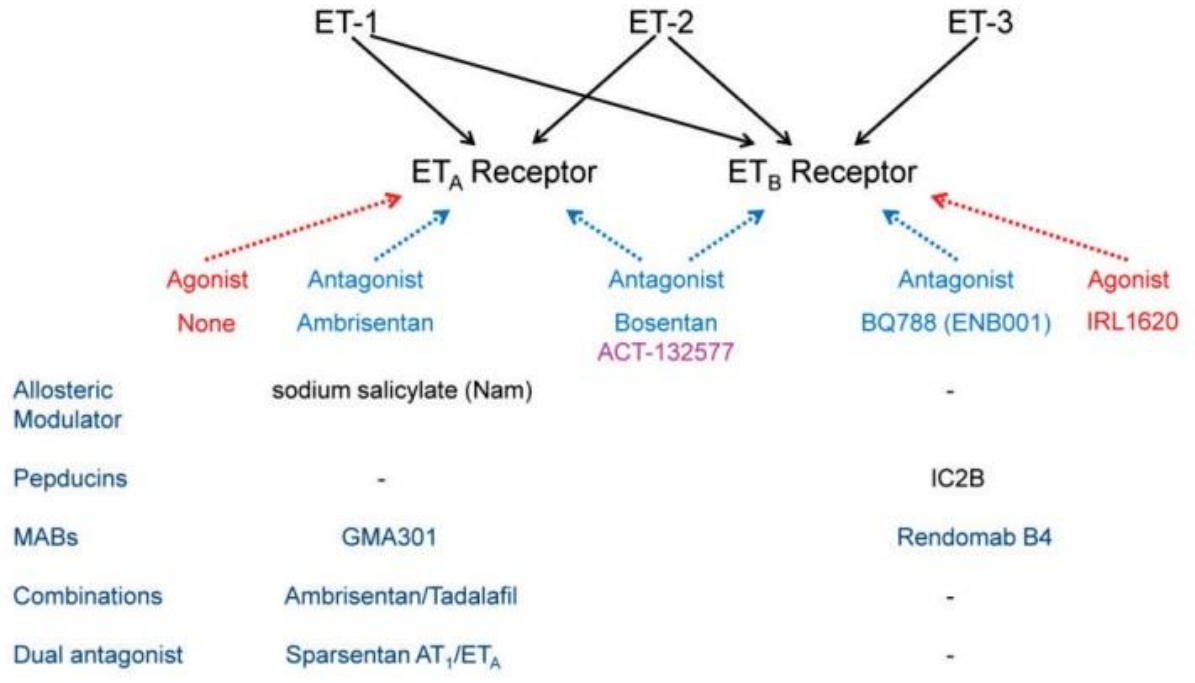


Figure 5: *Endothelin agonists and antagonists. (From reference **(26)**.)*

1.6 PHYSIOLOGICAL ACTIONS OF ENDOTHELIN-1

Administration of ETA receptor antagonists in healthy volunteers induces vasodilation 7. When administered systemically the selective ETA receptor antagonist BQ-123 caused a dose-dependent decrease in blood pressure and vascular resistance **(27)**. This fact indicates that ET-1 contributes to the maintenance of vascular tone and blood pressure through the ETA receptors. On the other hand, the selective ETB antagonist BQ-788 caused a mild vasoconstriction as well as a small increase in blood pressure **(28, 29)**. This suggests that ETB receptors, associated with NO (nitric oxide) synthesis, normally play a compensatory role to ETA receptors in maintaining vascular tone. Indeed, administration of a dual ETA/B receptor antagonist causes milder vasodilation and smaller reduction in blood pressure **(30)**. Plasma concentration of ET-1 in rodents and humans has been elevated by ETB antagonists, possibly by reducing its clearance through the ETB receptors **(9, 28)**. ET-1 also has other physiological properties, in addition to its vascular actions through the ET receptors (Fig.6). In particular, ETB receptors, in addition to their presence in the endothelial cells, where they induce vasodilation, they are also produced in the epithelial cells of the renal tubules. There, ETB receptors prevent the actions of vasopressin and inhibit Na/K ATPase, in response to ET-1 intrarenal synthesis, thus leading to salt and water loss **(31)**. This hypothesis is supported by the observation that mice lacking the gene for the ETB receptors have the phenotype of salt-sensitive hypertension **(32)**. Furthermore, ET-1 collection by the kidneys has been shown to be a physiological regulator of sodium excretion **(33)**. Finally ET-1 plays an important role in embryonic development through both types of receptors.

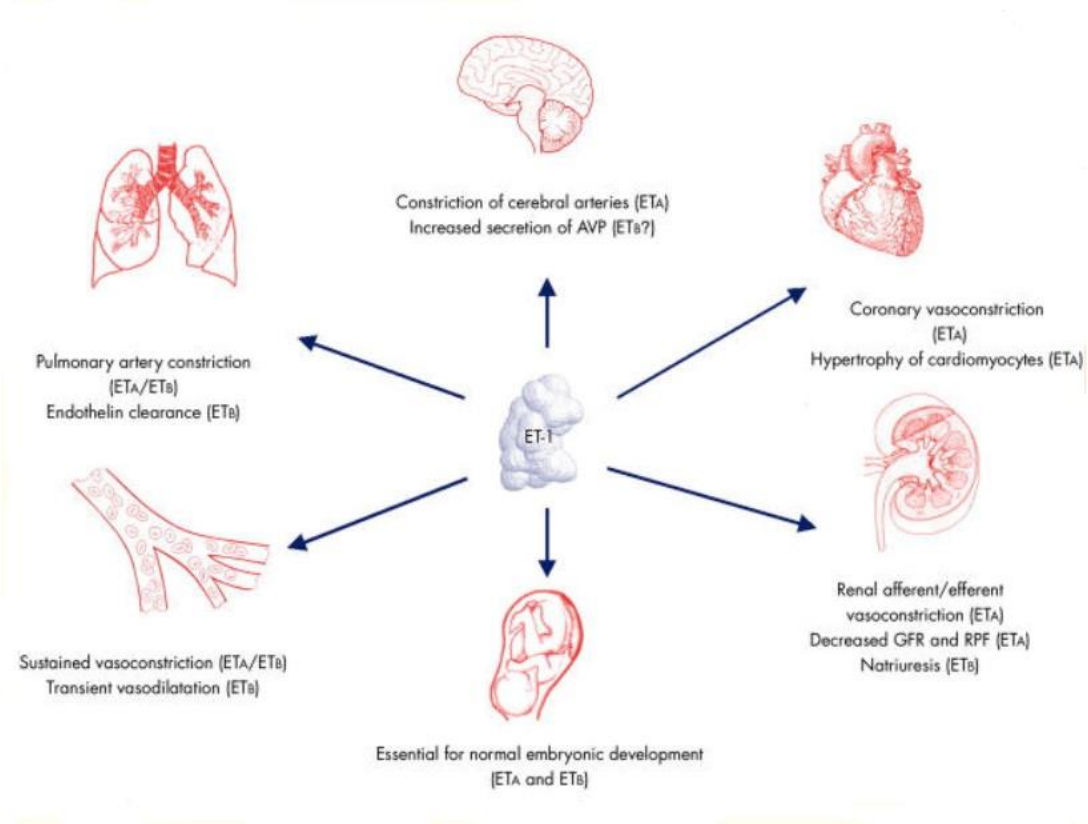


Figure 6: *Endothelin-1 (ET-1) actions. AVP, arginine vasopressin; GFR, glomerular filtration rate; RPF, renal plasma flow. (From reference **(34)**. With permission)*

2. ISCHEMIA AND MYOCARDIAL INFARCTION

Myocardial ischemia is defined as an insufficient blood supply to the myocardial tissue. The difference with myocardial infarction is that the second refers to myocardial tissue necrosis, which occurs after a prolonged period of ischemia (>20 minutes or less for some animal models) **(35)**. The criteria of the diagnosis of acute myocardial infarction, based on clinical, electrocardiographic and biochemical evidence, according to the 2018 guidelines **(36)**, are: when there is myocardial injury with clinical evidence of acute myocardial ischemia and with detection of rise and/or fall in the values of cardiac biomarkers (with preferably cardiac troponin, cTn) and presence of one of the following:

• Symptoms of myocardial ischemia;

• New ischemic ECG changes;

• Development of pathological Q waves;

• Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischemic etiology;

• Identification of a coronary thrombus by angiography or autopsy.

Acute coronary syndromes include myocardial infarction, unstable angina, and sudden cardiac death. The major cause of acute coronary syndromes is the rupture of an unstable atherosclerotic plaque in the coronary circulation, resulting in occlusion of the artery. Rupture of the atherosclerotic plaque and erosion of the endothelium lead to the gathering on the endothelium of activated platelets (white thrombus) and subsequently red blood cells and fibrin (red thrombus). A reduction of the blood flow or even complete cessation of the blood supply to the myocardium is caused, resulting in its ischemia **(37)**.

The major results of the infarction are left ventricular remodeling, which can lead to heart failure, and ventricular arrhythmogenesis, which can cause sudden cardiac death. Despite the strategy used to save the ischemic myocardial tissue, through reperfusion therapies, the number of patients with post-infarction heart failure remains notably large **(38)**.

2.1 PATHOPHYSIOLOGY AND HISTOLOGIC CHANGES DURING MYOCARDIAL INFARCTION

The duration of the coronary artery occlusion, the site of the occlusion (and therefore the spread of the ischemic area) and the existence of collateral circulation, are factors that define the final size of the infarct. Experimental studies have shown the beneficial effect, on the infarct size, of ischemic preconditioning of the myocardium (short repeated cycles of ischemia/reperfusion preceding the main ischemia) **(39)** as well as post-ischemic protection of the myocardium (short repeated cycles of ischemia/reperfusion following of the main ischemia) **(40)**. Lastly, myocardial oxygen metabolic needs is also a factor affecting the infarct size, although some studies question the importance of it **(41)**.

The first hours after the acute occlusion of the coronary artery, changes are observed in the myocardium. Macroscopically, congestion and pallor of the ischemic area are observed. Microscopically, the cytoplasm is disrupted and the myocardial fibers are separated, with leukocytes gathering between them at the same time.

In the following 24-72 hours, significant changes establish in the necrotic myocardium. Macroscopically, the necrotic myocardium acquires a yellowish color, while microscopically, neutrophilic influx and accumulation of macrophage white blood cells is noted in the necrotic myocardial fibers. On the 3rd to the 10th day, phagocytosis of the remaining parts of myocardial fibers is observed, while granulomatous tissue is formatted. Finally, weeks after, healing is completed, and white fibrous connective tissue replaces the myocardium (Fig.7). The myocardial area around the infarct is of particular interest, because surviving myocardial fibers are often present **(42)**.

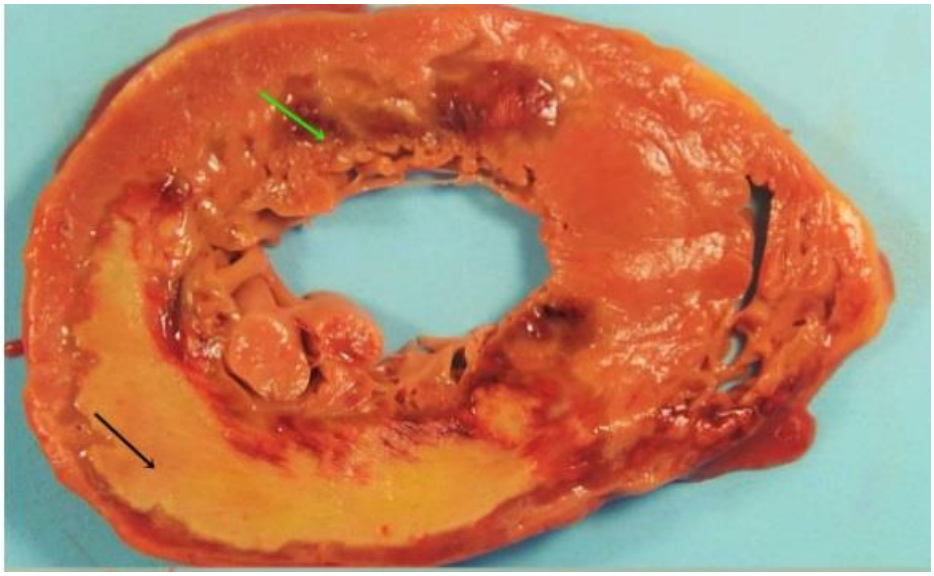


Figure 7*: Horizontal section of a heart with myocardial infarction. Green arrows point to dark mottling, and the black arrows point to a yellow, softened lesion with red-tan borders; these correspond to a myocardial infarction in between 12 to 24 hours and 10 to 14 days, respectively. (From reference* ***(42)****. With permission)*

2.2 HEART FAILURE

In the early stages after acute infarction, left ventricular dysfunction can be asymptomatic. The most common dysfunction following myocardial infarction, is heart failure (HF), which accounts for a large percentage of coronary heart disease deaths. Heart failure is defined as a clinical syndrome, which is characterized by specific symptoms and signs, caused by structural and/or functional abnormalities of the heart, resulting in a decrease of contractility and/or an increase of intracardiac pressures at rest or during exercise. The severity of HF varies and is defined by specific criteria. Thus, HF based on:

- the left ventricular ejection fraction (LVEF) is divided into A) HF with preserved LVEF (>50%) B) HF with reduced LVEF (<40%) and C) with intermediate LVEF (41%-49%) (Table 1)

- the time onset, is distinguished in chronic HF (established diagnosis of HF or more gradual onset of symptoms) and acute HF (rapid or gradual onset of symptoms/signs that lead to urgent medical care)
- the severity of the symptoms and the structural changes of the heart, the simplest terminology used is the New York Heart Association (NYHA) functional classification, is presented in Table 3 **(43)**.

Table 3. *Heart failure classification according to the NYHA. (From reference **(43)**. With permission)*

Class I	No limitation of physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.
Class II	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in undue breathlessness, fatigue, or palpitations.
Class III	Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity results undue breathlessness, fatigue, or palpitations.
Class IV	Unable to carry on any physical activity without discomfort. Symptoms at rest can be present. If any physical activity is undertaken, discomfort is increased.

All HF classifications have in common the inability of the heart to function effectively as a pump, supplying blood to the periphery and meet the body's metabolic needs. Heart failure is a progressive process, in the development of which neurohormonal mechanisms contribute. The main ones are the activation of the renin-angiotensin-aldosterone system and the stimulation of the sympathetic nervous system **(38)**. These mechanisms work countervailingly, to maintain an adequate supply of blood to the tissues, but their long-term presence is detrimental to the body.

2.2.1. Autonomic nervous system and heart failure

Activation of the sympathetic nervous system with a concomitant reduction of parasympathetic tone, take place in heart failure. This mechanism achieves a compensatory increase in myocardial

contractility and heart rate, thus directing the increased blood supply to the tissues. The increase in sympathetic and simultaneously the decrease in parasympathetic tone cause a decrease in heart rate variability (HRV) and an increase in the peripheral vascular resistance **(44)**. The stimulation of the sympathetic tone results in elevated concentration of norepinephrine in the myocardium, which causes a gradual decrease of β1 adrenergic receptors. Therefore, β2 to β1 ratio is increased in the failing myocardium **(45)**. Although norepinephrine improves myocardial contractility and dilation and maintains blood pressure and blood flow, its continuous effect may be harmful, as it increases myocardial metabolic oxygen requirements and may trigger ventricular arrhythmias, particularly during the myocardial ischemia.

2.2.2. Left ventricular post-infarct remodeling

The changes that occur in the failing myocardium can be categorized into those that happen in the myocardial cell and those in the extracellular matrix. Progressive loss of myocardial cells occurs through coagulative necrosis and apoptosis and contributes to left ventricular remodeling **(42)**. During post-infarction period, loss of cardiomyocytes results in segmental myocardial hypokinesis and progressive alteration of left ventricular architecture.

The infarcted area thins and elongates, and the left ventricle (infarct and non-infarct regions) undergoes progressive changes in its size and geometric shape. After myocardial infarction, developing alterations take place at molecular, cellular, and extracellular levels. Clinically, those changes translate into left ventricular shape, size, and function alterations. The set of these processes is called LV remodeling. Although remodeling appears to be a process of post-infarction adaptation of the left ventricle, aiming to preserve stroke volume and ejection fraction, it leads to overall left ventricular dysfunction, in the long term. Table 4 summarizes the processes taking place during LV remodeling **(46, 47)**.

Table 4. *Left ventricular (LV) remodeling processes.*

LV Remodeling
Thinning of the LV wall
LV dilation
Infarct area expansion
Inflammation
Myocardial cells phagocytosis
Accumulation of fibroblasts / Scar formation
Endothelial cell activation
Neoangiogenesis

LV remodeling is affected by changes:

1) in the inflammatory response (neutrophils and macrophages)

2) in the hemodynamic load

3) in the neurohormonal activation

4) in the extracellular matrix (fibrosis and activation of extracellular proteases, including metalloproteinases (MMPs) and serine proteases).

The extracellular matrix and its components participate in the progression of remodeling after infarction. The extracellular matrix is tissue-specific and has different quality and quantity amongst tissues. Extracellular matrix of the heart consists of collagen (types I, III, VI, V, and VI), glycoproteins (fibronectin, lamins, periostin, fibromodulin, and vitronectin), proteoglycans (versican, lumican, and diglycan), glycosaminoglycans (hyaluronic acid, and dermatan sulfate), and matrix proteins. The source of most of the extracellular matrix proteins is cardiac fibroblasts. Post-infarction, myofibroblasts, neutrophils, mast cells, lymphocytes and macrophages synthesize a series of proteins that participate in both the healing process and the remodeling process **(48)**.

3. ELECTROPHYSIOLOGY AND ARRHYTMOGENESIS POST-INFARCTION

3.1. ELECTROPHYSIOLOGICAL CHANGES AFTER ISCHEMIA

ION CONCENTRATION AND CURRENT OF INJURY

Ischemia primarily alters the extracellular and intracellular cationic concentration, of the myocardial cell. Specifically, inhibition of the Na^+/K^+ pump function reduces the intracellular concentration of potassium, and correspondingly increases its extracellular concentration. Moreover, the accumulation of lactic acid and the subsequent acidosis cause an increase in intracellular sodium concentration, via the Na^+/H^+ pump. This, ultimately results in an increase of the intracellular calcium concentration, via the Na^+/Ca^{2+} exchanger, which causes early and late depolarizations of the cell membrane action potential. This pathological calcium ion concentration causes an increase of the resting potential in the cardiomyocytes of the ischemic region, from -85 mV to -60 mV, resulting in the creation of a potential difference of the ischemic and healthy regions. As a consequence, the current of injury is created during dilation, specifically, electrical flow directed from the ischemic myocardium (higher potential) to the healthy myocardium (lower potential) **(49)**.

ACTION POTENTIAL

During ischemia the action potential is reduced in both duration and amplitude (Fig.8). There are cases where the duration increases in the very early phase of ischemia, but ends up decreasing sharply, soon after. The most likely pathophysiological mechanism for the duration shortening of the action potential is the opening of the ATP-sensitive potassium channels (KATP), which causes an increase of the outward K^+ current **(50)**.

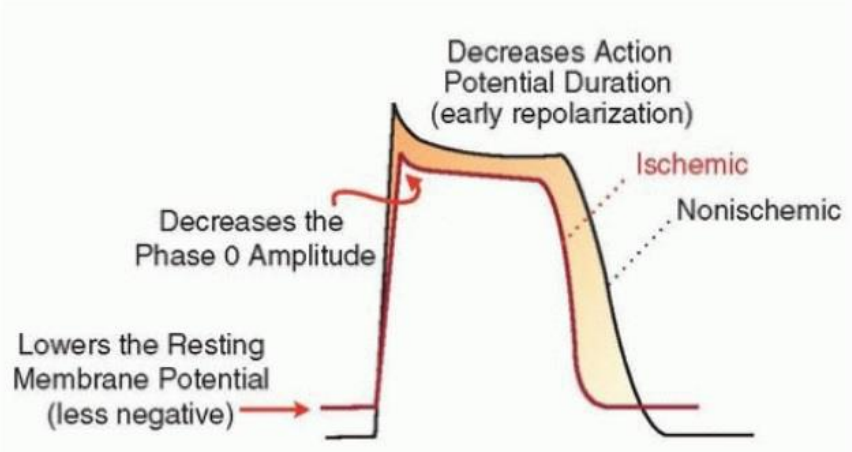


Figure 8*: Effects of ischemia in the myocardial action potential.*

A decrease in conduction velocity and depolarization velocity is observed, where it appears as a decrease of the action potentials slope in phase 0 **(51)**. Changes are also observed in the non-excitable period, of the ischemic myocardium. Even though the non-excitable period does not exceed the duration of the action potential, in the normal myocardium, during the first minutes of ischemia the non-excitable period decreases, but subsequently increases and extends when action potential ends. This phenomenon is known as post-repolarization inexcitability. Consequently, while the action potential is shorter in the ischemic myocardium in comparison to the normal, conduction velocity remains reduced due to the effect of post-repolarization inexcitability **(52)**. The decrease in the depolarization velocity and the prolongation of the non-excitable period are due to the partial deactivation of the sodium channels **(53)**. In the borderzone of the ischemic region, a decrease in both the non-excitable period and the duration of the action potential is observed **(52)**. These changes, as well as the heterogeneity of conduction between ischemic and healthy myocardium create a suitable substrate for the appearance of re-entry circuit and the genesis of ventricular arrhythmias.

Several minutes after the onset of ischemia (approximately 30 min), part of the ischemic myocardium has turned necrotic. In this region of the established infarct, the myocardium does not respond to any stimulus and no potential is generated **(52)**. In different regions of the ischemic myocardium, variation of the action potential duration is observed. This variation is called spatial differences (Fig.9). It has been noted that during the ischemia phase, there are also

time-related alternans in the amplitude and duration of the action potential of the same myocardial region (Fig.10) **(54)**.These alternans are actually morphologically different action potentials of a cell, periodically repeated. Spatial differences and changes in the potential over time underlie post-infarction arrhythmogenesis **(55, 56)**.

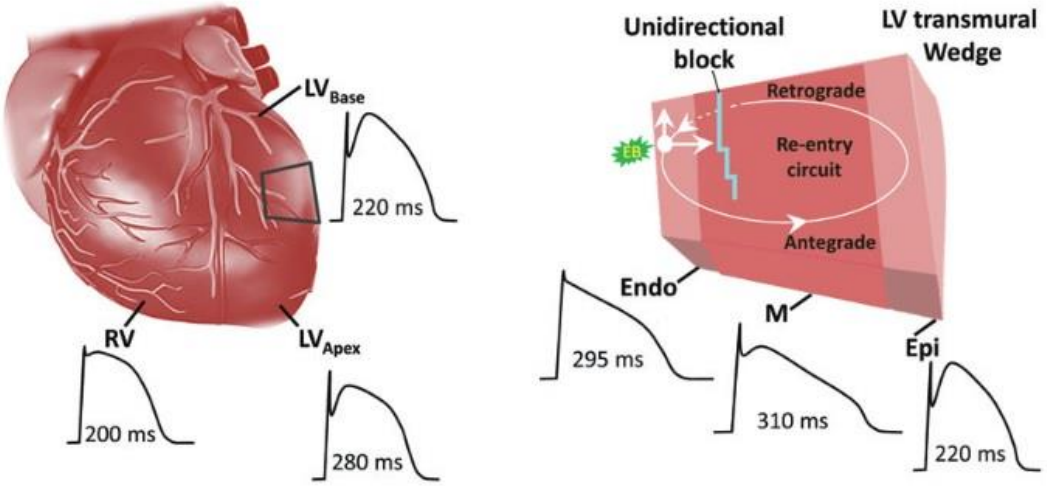


Figure 9*: (left) Variation of the action potential at the different regions of the myocardium (spatial differences). (right) Dispersion of action potential in the myocardial wall (endocardium-myocardium-epicardium). (From reference* ***(57)****. With permission)*

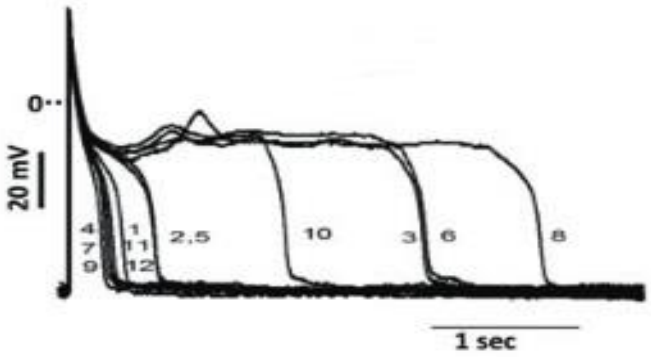


Figure 10*: Temporal differences in the duration of the action potential. (From reference* ***(57)****. With permission)*

ELECTROCARDIOGRAPHIC CHANGES

Electrophysiological changes caused by myocardial ischemia are recorded on the surface electrocardiogram, with the main feature being changes in the ST interval. Acute myocardial infarction with ST segment elevation on the electrocardiogram, accounts for approximately 50% of acute coronary syndromes **(58)**. When the electrocardiogram is obtained with a direct and current-enhancing electrode, facing the ischemic area, the fall in the resting potential is reflected in the initial TQ interval fall, during the first minutes of the ischemia **(59)**. On the surface ECG, this change is represented by an ST segment elevation (Fig.11). The endogenous deviation increases and the QRS wave widens. Five minutes after, the ST segment is further elevated due to the shorter action potential of the ischemic myocardium. Later, when activation of the ischemic region is significantly delayed, ST elevation becomes even more pronounced and is accompanied by a markedly inverted T wave. Eight to fifteen minutes after the ischemia onset, intraventricular conduction temporarily returns and is accompanied by T-wave alternans **(60)**.

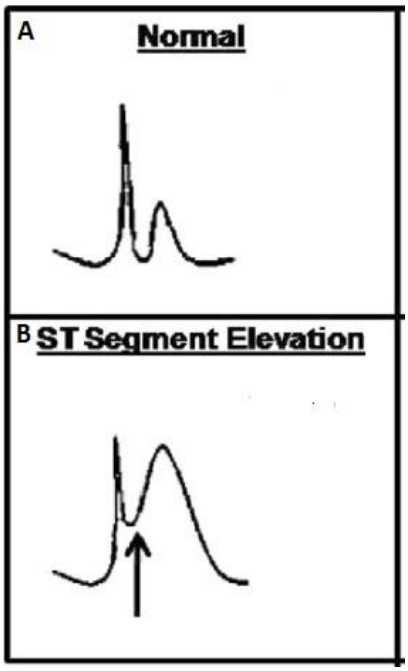


Figure 11*: (A) Normal ST segment and T-wave, (B) Progressive ST segment elevation with continued prominent T-wave. (From reference **(61)**. With permission)*

3.2. VENTRICULAR ARRHYTHMOGENESIS: MECHANISMS AND ARRHYTHMOGENIC FACTORS

3.2.1. Basic mechanisms

Arrhythmogenesis is caused by disturbances in the generation and conduction of electrical depolarization. The mechanical function, contraction/dilation, is precisely coordinated by the sequence of electrophysiological changes which constitute heart rhythm. Sinus rhythm is a prerequisite for the normal heart functioning, while rhythm disturbances can affect the smooth blood flow. These disorders can lead to health consequences, from mild and transient, to fatal. Consequently, understanding the mechanisms that may alter the physiological rhythm is essential in clinical practice, as it helps in the interpretation of the symptoms.

ELECTRICAL DEPOLARIZATION GENERATION DISTURBANCES

SINUS NODE PATHOLOGICAL AUTOMATICITY

The function of the sinus node as a pacemaker can be disrupted, resulting in abnormally low or extremely high depolarization frequency. During ischemia, the onset of arrhythmias often follows the change in the frequency. The frequency alteration is mainly due to neurogenic (ANS) and hormonal stimulation or due to ionic currents direct effect **(52)**.

ECTOPIC PATHOLOGICAL AUTOMATICITY

Spontaneous depolarization in ectopic centers is mainly due to the activation of the nonselective cation current, which is activated by hyperpolarization. Moreover, it is caused by the slow release of calcium ions from the sarcoplasmic reticulum, the activation of the Na^{+}/Ca^{2+} pump, and the activation of currents sensitive to wall stress. Pathological depolarization-induced automaticity exists predominantly in the intermediate zone between the healthy and the infarcted myocardium. Automaticity in the AV node, Purkinje fibers, atrial and ventricular cells can

influence the initiation of Ia phase arrhythmias and the initiation and maintenance of Ib phase arrhythmias **(52)**.

TRIGGERED ACTIVITY

Two types of triggered activity are distinguished: early afterdepolarizations and late afterdepolarizations. Early afterdepolarizations are secondary depolarizations that arise during phase 2 or 3 of repolarization of the action potential **(62)**. Delayed afterdepolarizations are transient depolarizations triggered by a complete or nearly complete preceding action potential **(63)**.

ELECTRICAL DEPOLARIZATION CONDUCTION DISTURBANCES

REENTRY MECHANISM

This mechanism consists in the repeated movement of an electrical stimulus in a myocardial tissue region (called the substrate), causing a corresponding depolarization of the rest of the (atrial and/or ventricular) myocardium. A reentrant stimulus is conducted along a myocardial pathway, but instead of unwinding and decaying, like a normal stimulus, it re-excites cells that were previously excited **(64)**. The reentry substrate can be a normal anatomical structure, such as the sinus node, but mostly it consists of pathological anatomical or histological structures in the left or the right ventricle. In some cases, a reentrant circuit may form around a myocardial region that differs from the rest of the myocardium not histologically, but in terms of its electrophysiological properties. This area does not consist of pathological tissue, but of myocardium, which becomes inexcitable, due to continuous depolarization by electrical impulses converging towards this area. Two conditions must be met to create a reentry circuit: (a) the existence of more than two electrical current pathways and (b) the combination of low conduction velocity and a short non-excitable period in the rest of the pathway.

In ischemia, these two conditions are fulfilled, since conduction velocity decreases and at the same time a great heterogeneity of the non-excitable period, occurs **(65)**. 15 min after the

ischemia onset, where disruption of gap junctions becomes more apparent and cells are uncoupled, the likelihood of reentry circuit and ventricular arrhythmia increases **(66)**.

3.2.2. Arrhythmogenic factors

VENTRICULAR REMODELING POST-MI

Key factors in the generation of ventricular arrhythmias during the acute phase of ischemia are changes in the ionic currents (increase in extracellular K^+ and intracellular Ca^{2+}) and dephosphorylation of connexin 43, since they lead to reduced cell coupling **(67)**. Moreover, the abnormal expression and distribution of connexins alter gap junction function and further compromise electrical conduction. Structural remodeling, as described before, is histologically characterized by fibrotic tissue, which is formed not only in the areas within and adjacent to the infarct, but also in remote myocardial areas. In the presence of fibrosis, areas of slow conduction provide the substrate for the formation of reentrant circuits.

In the chronic phase of infarction, the main arrhythmogenic factor is remodeling in the architecture of the left ventricle. This change consists of hypertrophic cells with disturbed coupling between them, change in the direction of fibroblasts and reduction of gap junctions. The above conditions, which lead to a reduction in conduction velocity and the existence of one-way blockade, create the substrate for the creation of reentry circuits **(67)**. This process is also known as electrical remodeling of the ventricles **(68)**.

INCREASED SYMPATHETIC DRIVE

The autonomic nervous system plays a catalytic role in arrhythmogenesis during the acute phase of the heart attack. It is well established that the autonomic nervous system consists of a complex neuronal set, which regulates different systems and aims at maintaining homeostasis by adapting to external and internal environmental changes. During exercise and emotional stress, the sympathetic nervous system provokes heart rate increase, as well as conduction velocity and contractility elevation. All those sympathetic-driven changes allow for the increased metabolic

demand during those conditions. In heart failure, increased sympathetic stimulation also occurs, as an adaptive response, enhancing healthy myocardium's contractility to counterbalance the loss of the ischemic region. However, disturbance of sympathetic and parasympathetic nervous system balance is associated with several maladaptive mechanisms that promote arrhythmogenesis in the long-term **(69)**.

During ischemia, the stimulation of the sympathetic nervous system increases with a concomitant decrease of the parasympathetic tone. This central sympathetic nervous system stimulation appears to alter the duration of repolarization in the myocardium **(70)**. Furthermore, the local catecholamine release modifies the electrophysiological properties of the myocardium, creating a functional substrate for arrhythmogenesis **(71)**.

Central inhibition of the sympathetic nervous system (with clonidine) has been found to reduce both the duration and the episodes of ventricular arrhythmias in phase II post-infarction arrhythmogenesis **(72)**. Many experimental studies, point out the autonomic nerve endings remodeling, which occurs in the ventricular myocardium post-infarction. This nerve remodeling affects both the ischemic and the healthy areas (sympathetic hypersensitivity) and leads to an inhomogeneous electrophysiological response of the myocardium in the early stages of acute ischemia **(73)**. The locally heterogeneous sympathetic innervation of the myocardium, increases repolarization dispersion and this dispersion is proportional to the denervation size of the ischemic area **(74)**.

On the contrary, parasympathetic nervous system appears to reduce arrhythmogenesis **(73, 75)**. The anti-fibrillation effect of the parasympathetic nervous system is described in many experimental studies and is due either to a direct action on muscarinic receptors and a reduction of the heart rate, or to the release of nitric oxide, which acts as a mediator **(75)**.

4. POST-INFARCTION ARRHYTHMIAS

Arrhythmias in the acute stage of infarction are classified according to their time of onset. Those that appear within the first 30 minutes of the ischemia are characterized as phase I arrhythmias, while those that appear within the following 72 hours are called phase II arrhythmias. Arrhythmias of the chronic stage of myocardial ischemia are characterized as phase III arrhythmias but since they are not the subject of this thesis, the pathophysiological mechanisms responsible for them will not be mentioned.

4.1. PHASE I ARRHYTHMIAS

Phase I arrhythmias include all arrhythmologic events that occur the first 30 minutes of ischemia, when irreversible cellular damage has not yet established. In dogs, pigs, sheep and rats this phase can be divided into two distinguished phases, Ia and Ib. A similar distinction in humans cannot be done with absolute certainty, but there are indications that humans also have an early and a late period of increased arrhythmogenesis after MI **(76)**.

Arrhythmias in phase Ia (2 to 10 minutes after the coronary occlusion) are usually ventricular tachycardias (VT) which rarely progress to ventricular fibrillation (VF). Mapping studies have shown that the mechanism of their generation are reentry circuits **(77)** , with the stimulus following a very long pathway through the ischemic tissue, or two stimuli traveling around an eight-shaped conduction block region. Arrhythmia onset is often preceded by alternating T waves and deep negative T waves, which are caused by significant differences in cell activation and repolarization.

The initial stimulus appears close to the ischemic borderzone. The nature of the initiating stimulus has been studied in cats and has been found to be transmural reentry in the 76% of cases and early depolarizations from the subendocardium or epicardium in the remaining 24%

(77). One of the local mechanisms causing those depolarizations is the excitation from the injury current. This current can cause re-stimulation of normal cells or even increased automaticity. During deep negative T-waves, the injury current is much bigger than it is in dilation. Not surprisingly, the initial tachycardia pulses often follow deep negative T-waves.

Ib phase arrhythmias (20 to 30 minutes after the coronary occlusion) are less studied. They are concurrent with a massive release of catecholamines, the onset of a significant increase in longitudinal resistance **(78)** and an increase in extracellular potassium and intracellular calcium. These changes favor reentry circuits. This phase is far more dangerous as a significant proportion of VTs which progress in VFs and sudden death, can occur.

4.2. PHASE II ARRHYTHMIAS

Arrhythmias of this phase occur after at least 90 minutes of ischemia. Phase II coincides with the initiation and completion of myocardial necrosis. This period is accompanied by several metabolic, ionic and electrophysiological changes. Metabolically, it is characterized by low intracellular levels of glycogen and high lactic acid, with cessation of anaerobic glycolysis and a reduction of ATP and creatine phosphate **(79)**. In addition, the adenine nucleotide pool consists mainly of AMP 98. Regarding the ionic changes, the accumulation of extracellular potassium that began in phase I continues in this phase as well. Conversely, intracellular potassium levels decrease. Furthermore, high concentrations of intracellular sodium and calcium are observed.

The primary source of arrhythmias in this phase is thought to be the surviving but dysfunctional Purkinje fibers **(80, 81)**. After one hour of ischemia, the surviving Purkinje fibers and myocardial fibers show reduced resting potential and action potential velocity. Action potential is shortened in Purkinje fibers, while it is lengthened in the myocardial fibers, thus favoring reentry **(82)**. While reentry and injury current account for phase I arrhythmias, reentry and abnormal automaticity (delayed afterdepolarizations) appear to predominate during the infarct completion. On the other hand, there are no significant differences in the ECG and hemodynamic parameters that describe the few seconds preceding the ventricular arrhythmias in either of the phases **(76)**.

Not well understood is also the absence of arrhythmic episodes between the two phases **(83)**, as well as the cessation of their appearance after 8 to 10 hours. Possible explanations are the eventual loss of excitability of cells in the core of the ischemic area and the completion of cell necrosis despite the ongoing inflammatory reaction **(83)**.

5. ENDOTHELIN AND ACUTE MYOCARDIAL INFARCTION

5.1. ENDOTHELIN IN HEART FAILURE

Chronic heart failure is a very severe cardiovascular disease, which can often lead to high rates of morbidity and mortality. In most cases it is characterized by low cardiac output, which progressively leads to hemodynamic and neurohormonal adaptations of the body, such as peripheral vasoconstriction, water and salt retention, and activation of the renin-angiotensin and sympathetic systems **(84)**. Among the activated systems during this condition is the endothelin system. Plasma concentrations of big ET-1 and ET-1 have been associated with clinical and hemodynamic factors of particular severity for patients with chronic heart failure and are inversely related to the prognosis of these patients **(85-87)**.

Additionally, while in the normal heart ET-1 appears to exert a positive inotropic effect, in the failing heart the administration of ETA receptor antagonists of ET-1 has been reported to increase its contractility, reflecting a negative inotropic effect **(88)**. Increased ET-1 levels and altered cardiac, vascular, and pulmonary ET-1 receptor characteristics have been clearly described in heart failure studies **(89-92)**. Furthermore, the positive effects from the combination of ET-1 receptor antagonists and endothelin converting enzyme inhibitors in experimental and clinical heart failure studies confirm the role of ET-1 in the development of cardiac hypertrophy and functional failure **(92-96)**. However, despite the fact that all studies confirm an increase in ET-1 immediately after acute myocardial infarction, the role of this increase during the acute phase remains unclear.

5.2. HEART AND THE ENDOTHELIN SYSTEM

ET-1 has a positive inotropic and chronotropic effect **(6)**. However, when administered systematically in large doses, it acts as a strong vasoconstrictor of the coronary vessels, leading to a cardiac output decrease. High-dose intracoronary administration of ET-1 can be fatal because of the ischemia it causes. Conversely, when co-administered with vasodilators cardiac output increases, probably because its inotropic effect is revealed **(97)**. In normal hearts, cardiac contractility is reduced when ET-1 activity is inhibited **(88)**. In addition, ET-1, when acting through ETA receptors, regulates the secretion of atrial natriuretic peptide (ANP). Moreover, various studies suggest that ET-1 may have antiarrhythmic properties, especially during ischemia **(98-100)**. However, other studies suggest that ET-1 has proarrhythmic properties **(97, 98)**.

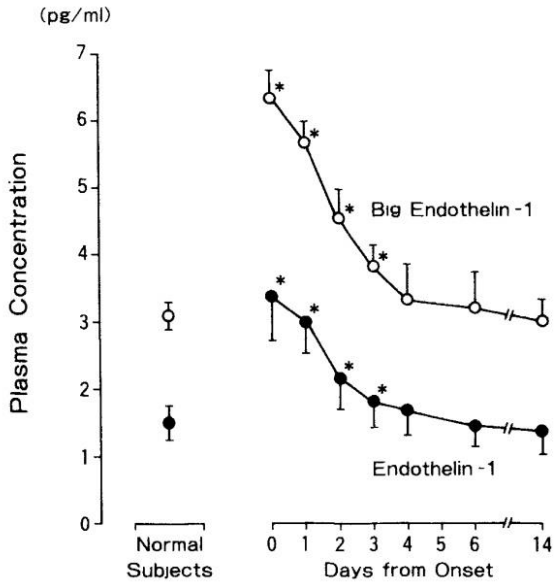
Expression of ET-1 in the normal heart is relatively low when compared to other organs, such as the kidney and lung **(101-103)**. ET-2 is present at much lower levels than ET-1, while ET-3 is not detected at all **(102)**. The cellular distribution of ET-1, as shown by immunohistochemistry, is characterized by its very high expression in the endothelium of the coronary arteries, followed by perivascular tissues and cardiac fibroblasts **(102, 104)** and by relatively low expression in the myocardial cells. Relative to their total mass though, a large proportion of cardiac ET-1 is found in the myocardial cells.

ET-1 receptors are found in the ventricular myocardium, in the atria and with a higher density in the AV node **(105)**. In the human ventricles the ETA/ETB ratio is 60/40 **(102)** but in isolated myocardial cells the ETA receptors percentage was found greater than 85% **(106)**. In normal rat hearts the percentage of ETAs has been also found greater than 80% **(107)**. Despite their low expression in the ventricles, ETB receptors appear to mediate the inotropic effect of ET-1 and facilitate coronary vasoconstriction under conditions of heart failure **(108)**. In addition, ETB receptors appear to play a role in hypertrophy-induced myocardial fibrosis during hypertension. **(109, 110)**.

5.3. ENDOTHELIN LEVELS IN MYOCARDIAL INFARCTION

PLASMA LEVELS

Shortly after endothelin discovery, it has been reported that an ischemic event results in increased ET-1 levels in the blood flow **(111-113)** and high expression of its binding sites **(114, 115)**. Miyauchi et al. **(112)** reported in 1989 that only a few hours after acute MI, both ET-1 and big ET-1 levels are elevated (Graph 1), while Stewart et al. **(116)** in 1991 found an early increase in ET-1 levels (even earlier than creatine kinase elevation) that peaks at 6 hours and returns to normal 24 hours after myocardial infarction. Conversely, in patients with complications, such as pulmonary edema, restenosis or cardiogenic shock, plasma ET-1 levels remained elevated for at least 72 hours post-infarction. In fact, the highest levels of ET-1 in plasma have been observed in patients with post-infarction cardiogenic shock **(103)**. These observations highlighted the clinical importance of ET-1 in acute myocardial infarction, which was reinforced by the study of Omland et al. **(117)**. In this study, ET-1 plasma levels three days after acute MI, were found to be a strong and independent predictor of survival of these patients one year later. In addition, studies on the big ET-1 precursor confirmed the importance of the ET-1 system in risk stratification of patients who have suffered an acute cardiac event **(85)**.



Graph 1*: Concentrations of endothelin-l and big endothelin-1 in peripheral venous plasma from patients with acute myocardial infarction. Mean (SE). Data for normal subjects were obtained from ten age-matched healthy volunteers. Plasma concentrations of endothelin-1 (e) or big endothelin-1 (0) at day 14 are not significantly different from those of normal subjects. Asterisks indicate significant difference from levels of day 14 (p < 0-05, paired t test). (From reference **(112)**. With permission)*

However, the origin of these elevated plasma ET-1 levels remains unclear. There are studies leading to the conclusion of this increase coming from the ischemic myocardium. Specifically, it has been reported that in *in vitro* ischemic conditions the heart releases ET-1 **(113)**, while in an experimental infarct-reperfusion model in rats, plasma ET-1 increased 50 minutes after the occlusion and this increase was enhanced upon reperfusion **(114)**. It should be noted that in chronic heart failure the ET-1 clearance from the lungs is reduced, a fact contributing to its plasma increase **(114, 118, 119)**. However, the extent of the relation of these observations to the acute phase of myocardial infarction, remains unclear.

CARDIAC LEVELS

There is also sufficient evidence from experimental studies, to believe that heart-derived ET-1 levels are significantly increased in the early phase of infarction. In the first related study, Watanabe et al. **(114)** reported that the ratio of cardiac ET-1 was significantly increased 1 h after coronary artery occlusion. Moreover, increased tissue expression of ET-1 has been found in the infarcted area of rat hearts. Peak expression of ET-1 coincides with peak plasma levels of markers of chronic inflammation, such as interleukin I (IL-1), TNF-a, and TGF-b, which are known to increase ET-1 activity. This fact indicates that ET-1 plays an important role in post-infarction myocardial inflammation and left ventricular remodeling **(120)**. An equally important finding of this study is also the increase in wall stress and ET-1 expression of the distal region. This finding suggests that a wall stress increase leads to an increase in ET-1 levels in both acute and chronic post-infarction heart failure **(120)**.

The mechanisms of the ET-1 increase immediately after acute myocardial infarction, remain unclear. The main stimulus appears to be myocardial ischemia, which through the activation of transcription factor 1-alpha **(121)**, probably activates ET-1 gene expression in myocardial cells. Furthermore, angiotensin II that increases under these conditions has been found to provoke ET-1 increase. Angiotensin II upregulates ET-1 expression in various cells **(122, 123)** and ETB receptors expression in myocardial cells **(124)**.

5.4. EFFECTS OF ET-1 DURING ACUTE MYOCARDIAL INFARCTION

5.4.1. Myocardial necrosis

There is insufficient evidence to support a significant effect of ET-1 on myocardial necrosis in the permanent coronary occlusion **(125)**. That is because three studies in the rat model reported no difference in infarct size when examined either selective ETA receptor blockade **(126)** or nonselective ET-1 receptor blockade **(127, 128)** in the setting of permanent ligation. In contrast, ET-1 has been found to promote inflammation and oxidative stress **(129)**, in the presence of reperfusion, since it is involved in the activation and accumulation of neutrophils **(130)** and in the release of cytokines from monocytes and macrophages. Studies using ETA receptor antagonists, in the rat ischemia – reperfusion model, ameliorated reperfusion injury by reducing infarct size and improving LV hemodynamics **(131-134)**. These results were confirmed in a small-scale clinical study, where short-term selective ETA receptor antagonist infusion prior to coronary occlusion improved myocardial reperfusion, decreased infarct size, and succeeded a small but significant improvement in LV function **(135)**.

5.4.2. ET-1 and central autonomic system

Factors regulating the central autonomic system during myocardial infarction are notably complex and are still not completely understood. In addition to myocardial and adrenal sites, growing evidence, based on ET-1 wide distribution in the brain and spinal cord of experimental animals **(136)** and humans **(137)**, suggest that the endothelin system also modulates central autonomic inputs. In fact, after intracisternal administration of endothelin, studies have described, potent hemodynamic changes **(138)**. The non-vascular location pattern of the endothelin receptors in the brain points towards neuropeptide modulatory actions **(139)**, which are likely mediated by changes in neuronal conduction and calcium influx

(140). Further support to the mechanism of the autonomic central regulation exerted by the brain endothelin system, have been provided by anatomical and functional studies **(141)**. For instance, measurements of the tyrosine hydroxylase activity have shown the interaction between endothelin and the olfactory system **(142)**. Furthermore, studies measuring cellular c-fos expression revealed the activation of the brainstem after administrating endothelin intracerebroventricularly, an action which is mediated by endothelin receptors **(143)**. Finally, sympathetic responses of the heart were modulated when endothelin was administrated in the paraventricular nucleus of rats; these dose-dependent effects were intercepted with ET receptor blockade **(144)**.

To further evaluate the role of the brain endothelin system in myocardial ischemia, a research group investigated the results of intracerebroventricular endothelin receptor blockade in rats with myocardial infarction **(72)**; this research targeted the endogenous endothelin system and avoided the distracting effects of administrating exogenous endothelin. The results revealed beneficial effects on delayed post-infarct arrhythmogenesis, whereas infarct size remained the same. Thereafter, the research team extended the observation period to also include early post-infarct arrhythmogenesis. Results showed decreased sympathetic activity, based on noninvasive indices derived from heart rate variability analysis, with an improved autonomic function, which was associated with a lower incidence of VTs during both arrhythmogenic phases **(145)**.

There is currently enough data to conclude that endothelin system-mediated autonomic regulation includes vagal responses in addition to the sympathetic component. Early research has shown that endothelin receptors are also present in the brainstem's dorsal vagal complex **(146)**, and that endothelin intracisternal injection causes vagal activation **(147)**. Following selective endothelin microinjections into the dorsal vagal complex of anesthetized rats, these findings were further verified. This intervention altered heart rate, arterial blood pressure, and gastric motor activity, with effects mediated by the ETA receptors **(148)**. These results are consistent with the study stated above **(145)**, which reported a slightly increased vagal activity following intracerebroventricular ET-receptor blockage.

5.4.3. Arrhythmogenesis

ET-1 exerts direct and indirect electrophysiologic effects (Fig. 12) and contributes to post-infarct ventricular arrhythmogenesis.

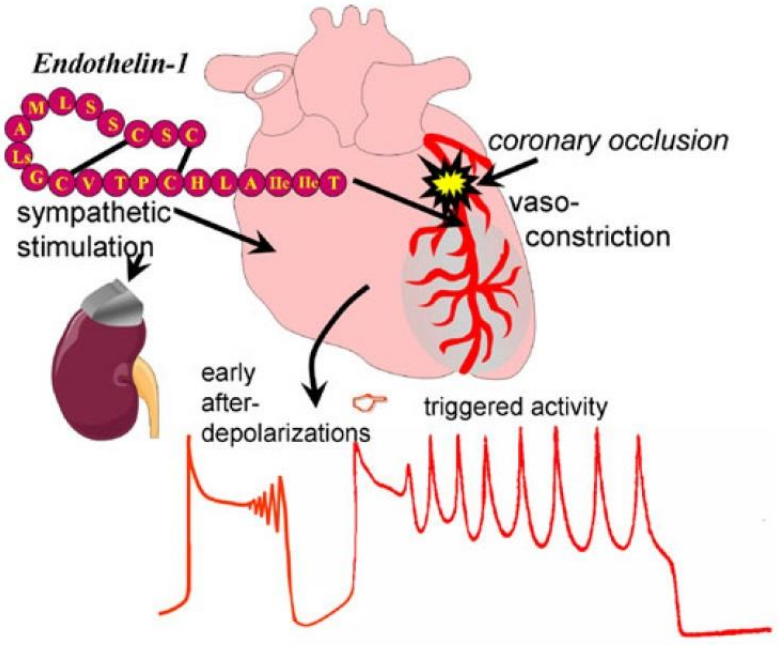


Figure 12*: Arrhythmogenic effects of endothelin-1 (From reference **(149)**. With permission)*

DIRECT EFFECTS

Endothelin exerts arrhythmogenic effects in isolated ventricular cardiomyocytes, consisting of early afterdepolarizations and enhanced automaticity **(150)**. A number of cellular mechanisms regulating these actions have been suggested, including activation of the Na^+/H^+ exchanger, rapid Ca^{2+} release from the sarcoplasmic reticulum through inositol trisphosphate receptors, or inhibition of delayed rectifier K^+ current **(149)**. Additionally, endothelin may deteriorate the gap junctional coupling of myocardial cells, thus contributing to anisotropic conduction **(151)**. This mechanism has been suggested in cellular electrophysiology studies, but its importance during myocardial ischemia is obscure. Of note, to support these finding,

scholars analyzed local activation of the ventricular myocardium, by using multi-electrode array recordings, in an *in vivo* preliminary study **(152)**. Since this mechanism could be potentially important, further investigation is required on the direct effects of endothelin on the electrical conduction in the ischemic myocardium.

INDIRECT EFFECTS

In addition to the direct arrhythmogenic effects of endothelin, research data have demonstrated a complex interaction between sympathetic nervous system activation and the endothelin system **(153)**. The interaction of the two systems takes place both at the adrenal gland level and at the ventricular myocardial level, with endothelin receptors exerting significant, albeit opposing effects **(125)**.

6. ACUTE EMOTIONAL STRESS AND AUTONOMIC NERVOUS SYSTEM

6.1. EMOTIONAL STRESS

Most people believe that stress results from an imbalance between the demands of the environment and a person's capacity to meet those expectations. Stress isn't just a result of the environment; it's also the result of the interaction between a certain external environment and a specific individual. As a result, not everyone will perceive and respond to a situation in the same manner. An event must cause a mental load which will strain the mind's ability to operate in order to be psychologically stressful. Psychological stress, to a significant level, arises whenever freshly obtained information does not easily fit into the existing pattern of knowledge previously stored in our memory **(154)**. Experience enables us create a mental image of who we are, the world in which we live, and how those two things relate to one another as we travel through life. When presented with new knowledge about the world around us, this mental image serves as a guide that directs our decision-making **(155)**. When new knowledge conflicts with the predetermined mental pattern that we have grown to believe is a reliable and accurate depiction of the outside world, stress arises.

Although both positive and negative changes in our surrounding necessitate modifications, published evidence support the idea that only unpleasant changes result in emotional stress reactions **(156)**. Physical stress including exercise, starvation, or cold, do not trigger the neuroendocrine cascade linked to psychological stress, if emotional arousal can be prevented. Surprisingly, despite humanity's ability to handle heat, cold, hunger and intense physical activity, we are nonetheless vulnerable to anxiety brought on by dissatisfaction or by anything that can jeopardize our safety and well-being. We can feel stressful emotional arousal due to stimulation of the limbic regions in the brain without being starved, wet, thirsty or cold **(157)**.

6.2. ACUTE TRIGGERING OF SUDDEN CARDIAC DEATH BY EMOTION

Anecdotal reports of people dying abruptly while experiencing intense emotion date back to the Bible, as Engel noted in 1971 **(158)**. Evidence that stress, anger, fear and other negative emotions can cause ventricular arrhythmias and sudden death is accumulating in demographic, clinical, and mechanistic studies. Studies on the epidemiology of population stresses, like earthquakes and war, have shown that acute psychological stress may lead to sudden death. Deaths during the Northridge earthquake in 1994 were studied and the data provided some of the early information on the effects of stress. There were six times as many sudden cardiac deaths on the earthquake's day as there were the day before and the day after. The fact that nearly all of these (21/24) were not caused by physical activity, suggests that the disaster's psychological consequences were the factor that caused the increased mortality **(159)**. A few years ago on 2011, the Great East Japan earthquake and tsunami increased the number of out-of-hospital cardiac arrests, not just in the most severely impacted regions **(160)**, but also in locations that were not directly affected **(161)**, demonstrating the negative effects of psychological stress. Similar to indirect effects, war also increases mortality by sudden cardiac death **(162)**. Even minor catastrophes have the power to cause abrupt death. For instance, daily cardiac death rates varied with daily changes in stocks, according to the analysis of daily death and stock performance data from Shanghai's Center for Disease Control and Prevention and Stock Exchange, respectively **(163)**. In demographic studies, even World Cup soccer matches have been linked to an increase in the risk of sudden death **(164)**.

6.3. AUTONOMIC RESPONSES ELICITED BY ACUTE EMOTIONAL STRESS

Acute emotional stress can trigger various autonomic responses in the body (Fig.13). Involuntary body processes like heart rate, blood pressure, digestion, breathing, and hormonal secretion are controlled by the autonomic nervous system (ANS). When a person experiences acute emotional stress, the ANS responds by activating the "fight-or-flight" response. This response is a conserved mammalian response whereby stress, fear or exercise elicits the activation of the sympathetic nervous system **(165)**. As a result, important cardiovascular alterations such enhanced cardiac chronotropy, lusitropy, and inotropy take place, which collectively cause a quick rise of the cardiac output **(166)**. The "fight-or-flight" response can also be linked to triggered activity, electrophysiological abnormalities and the initiation of fatal arrhythmias **(167)**, especially in the context of underlying cardiovascular disease, even if the "fight-or-flight" response is required to meet physical demands **(168)**. The sympathetic nervous system mediates these actions, through noradrenaline (mostly secreted from the cardiac sympathetic nerves) and adrenaline (released from the adrenal medulla) which bind to β-adrenergic receptors on cardiomyocytes and trigger the "fight-or-flight" response **(166)**. However, excessive sympathetic activity enhances automaticity and alters ventricular repolarization, thereby creating an arrhythmogenic milieu in the ventricular myocardium; on the other hand, excessive vagal activity leads to bradycardia and hypotension, often causing syncope **(169)**.

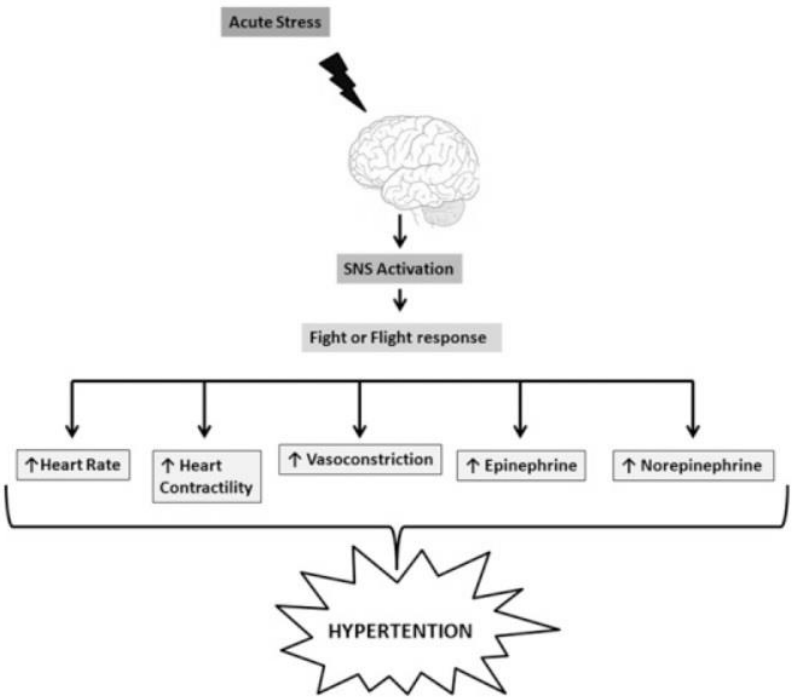


Figure 13*: Acute emotional stress can trigger various autonomic responses in the body. (From reference **(170)**. With permission)*

Hypothalamic-pituitary-adrenal (HPA) axis activation and a sympathoadrenal response, which involves fast stimulation of sympathoneural and adrenomedullary components, occur in response to stress, whether it is actual or anticipated. Direct nerve recording (sympathoneural component) or catecholamine spillover (humoral component) are two methods that can be used to measure baseline sympathetic activity or sympathetic reactivity in human as well as animal models **(171)**. The first assesses spikes in sympathetic nerve activity **(172)**, whereas the second assesses endogenous catecholamine secretion, typically noradrenaline **(171)**. Enhanced heart rate, peripheral resistance, cardiac output, urinary and plasma noradrenaline levels, as well as localized noradrenaline alterations, such as in the cardiac tissue, are all indicators of enhanced sympathetic stimulation **(173)**. Emotional stressors, whether actual or anticipated (anxiety), cause physiological reactions that, when abnormal, can be harmful to health.

6.3.1. Hypertension

It has long been believed that stress plays a role in the emergence of hypertension. Hypertension, which develops as a result of stress, is generally acknowledged to be caused by increased sympathoadrenal activity, increased release of norepinephrine and epinephrine, and higher vascular tone **(174)**. The sympathetic activation caused by acute emotional stress increases peripheral resistance and cardiac output **(175, 176)**. Notably, patients with essential hypertension exhibit stress biomarkers **(177)**. Hence, continuous and abnormal sympathetic stimulation as a result of emotional stress undoubtedly contributes to the mechanism behind the vast and increasing number of hypertension patients. It is well known that continuous and aberrant increases in sympathetic activity brought on by mental stressors at work or city noise can cause arrhythmogenesis and hypertension **(178-180)**. Inhibiting the stimulated SNS offers therapeutic advantages for the treatment of diseases, such as heart failure **(181, 182)**. On the contrary, prolonged vasoconstriction brought on by SNS activity cannot be stopped by total inhibition of the adrenergic receptors. Thus, it has been proposed that catecholamines are not the exclusive mediators of vasoconstriction through the SNS **(183)**.

6.3.2. Arrhythmogenesis

It is generally considered that acute emotional stress can lead to sudden cardiac death through alterations in sympatho-vagal balance; this notion is based on a wide range of experimental studies **(184, 185)** demonstrating the proarrhythmic potential of sympathetic stimulation or depressed vagal responsiveness. Enhanced dispersion of ventricular repolarization may be the end-result of these actions, manifested as T-wave alternans in recordings prior to ventricular fibrillation in animal-models **(186)** or patients with implanted defibrillators **(187)**. Comparable effects of stress on ventricular arrhythmias not related to ischemia have been shown in studies of patients with implantable cardioverter-defibrillators,

which restore potentially fatal arrhythmias to sinus rhythm by delivering a shock or anti-tachycardia pacing while saving information about the event. A case report, in which a patient who got the device for primarily preventive purposes, revealed a first-ever episode of ventricular tachycardia, shifted with anti-tachycardia pacing, of which the individual was unaware, and which took place while a family member's casket was being buried into the ground **(188)**. Increase in arrhythmogenesis treated by implantable defibrillators is also correlated with population stressors. For instance, not just in New York City **(189)** but also in Florida **(190)**, the attacks on the World Trade Center on 2001 were linked to a twofold rise of ventricular arrhythmias during the first thirty days that followed the attacks. Moreover, following the Great East Japan earthquake, a comparable rise in tachyarrhythmias in patients with implantable defibrillators was reported **(191)**. Finally, during the World Cup soccer games, the incidence of ventricular arrhythmias **(192)** increased as much as myocardial infarction **(193)** and sudden death **(164)**.

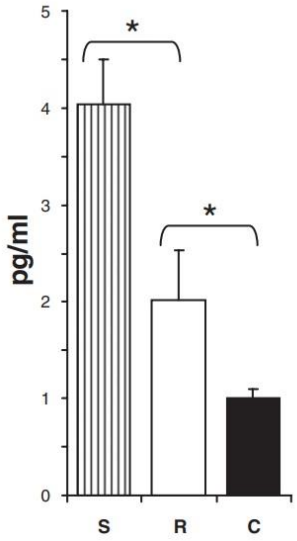
Electrophysiology studies have further shown how negative emotions might facilitate the generation of arrhythmias. Lown et al. examined in 1973, the arrhythmogenesis using programmed stimulation in a dog experimental model, in one of the first experiments to assess the role of stress in arrhythmias **(194)**. Stress was induced by lifting the dog in a sling which was conditioned to be an unpleasant stimulus. Only one PVC could be generated, with a high output of 35 mA, in rested animals, while by using only 5 mA, two PVCs were elicited during stress. In a human study, the impact of emotional stress on arrhythmogenesis in patients with a documented history of ventricular arrhythmias who also had implantable defibrillators, was assessed **(195, 196)**. All patients previously had VTs that ended with anti-tachycardia pacing. Arrhythmias driven by mental stress were more rapid and difficult to terminate than those induced during rest periods. Occasionally, an analogous VT that had been pacer-terminated in the baseline period, required shock for terminating during emotional stress. Thus, indicating that autonomic alterations brought on by stress had altered the VT circuit's characteristics of refractoriness and conduction, removing the excitable gap. Yet, despite the wealth of existing information, the factors governing autonomic discharges in response to acute stress are unclear. Furthermore, the mechanisms

by which strong emotional stimuli may alter ventricular electrophysiology and trigger ventricular tachyarrhythmias remain incompletely understood.

7. THE ENDOTHELIN SYSTEM AND ACUTE EMOTIONAL STRESS

7.1. ENDOTHELIN LEVELS IN RESPONSE TO ACUTE EMOTIONAL STRESS

Over the past few decades, scientific attention has been drawn to the mechanisms by which physiological reactions to stress contribute to the development of cardiovascular disease. Acute psychosocial/emotional stress boosts rises endothelin levels **(197)** while decreasing vagal activity in rats **(198)**. Surprisingly, similar results were obtained from numerous studies, which have shown that healthy or at-risk humans' plasma ET-1 levels rise in response to acute laboratory psychosocial stress **(199-202)**. Endothelin-1 has been identified as an emotional stress-responsive factor that may contribute as a mechanistic mediator of the connection between stress and cardiovascular disease. According to a systematic analysis of 20 studies, in both people at high risk for coronary artery disease and patients with prior acute coronary syndromes, acute psychosocial stress may evoke exaggerated plasma ET-1 release **(203)**. Moreover, in a population study, acute psychosocial stress-induced acute coronary syndrome was also associated with high elevation of plasma ET-1 levels. In this study the researchers found that when compared to similar patients without apparent sympathetic activation as a precipitating factor, patients admitted with acute coronary syndrome brought on by emotional stress had plasma ET-1 levels that were two times higher (Graph 2). Significant differences in plasma ET-1 concentrations between the designated clinical groups or among men and women were not detected **(204)**. Although the source of the circulating endothelin in these situations is unknown, the vascular endothelium seems to be the most likely cellular source **(205)**.



Graph 2: *Serum concentration of endothelin (ET)-1 in patients with acute psychosocial stress-induced acute coronary syndrome (S [ruled bar]) compared with reference patients with coronary syndrome but without apparent sympathetic activation (R [open bar]) and healthy control group (C [solid bar]). Bars and vertical lines represent mean SD values. *Significance between S, R, and C. The study population had a two-fold elevation on ET-1 levels than the reference patients, who had significantly higher levels than the healthy volunteers. (From reference **(204)**. With permission)*

7.2. AUTONOMIC RESPONSES DURING ACUTE EMOTIONAL STRESS

The response to cognitive and emotional stressors is, as mentioned before, characterized by increased sympathetic nervous system activity, as measured by a boost in catecholamine levels in the bloodstream **(206)**, and decreased parasympathetic nervous system activity, as measured by lowered heart rate variability in the high frequency domain **(196, 207)**. All of these reactions cause an increase of the heart rate, systolic and diastolic blood pressure **(208)**, and in patients with coronary artery disease (CAD), a decrease in myocardial blood flow because of the microvascular dysfunction **(209)** and epicardial vasoconstriction **(210, 211)**. Laboratory emotional stress also causes endothelial dysfunction, which can last for a period of time exceeding 90 minutes after the stress has ended **(212)**. Endothelin-1 (ET-1) plays a key part in this, as was recently shown by the ETA receptor blockade's ability to completely reverse this effect **(213)**. ET-1 is thought to be modulated during emotional stress by autonomic pathways, but not much are known about the mechanisms that mediate those actions. In a study including CAD patients, anger-recall stress caused an increase in

sympathetic activity and a decrease in parasympathetic activity, as was expected. However, only the parasympathetic withdrawal predicted a related increase in ET-1 and this increase is consistent with the cholinergic anti-inflammatory pathway **(214)**. In addition, several lines of evidence derived from experimental data have demonstrated a complex interplay between ET-1 and sympathetic activation, with ETA- and ETB-receptors exerting opposing effects **(214, 215)**. Research on the role of endothelin-1 in the rapid increase of blood pressure that occurs after psychosocial stress, in mice, specified endothelium-derived ET-1 and subsequent ET-A receptor activation as a novel mediator of the blood pressure response to acute psychosocial stress **(205)**. Importantly, high endothelin levels have been also associated with vagal dominance in neurocardiogenic syncope, characterized by bradycardia and hypotension, which is commonly observed after acute emotional stress (AES) **(216)**. The modulation or the regulating effects of ET-1 during acute emotional stress, while likely to involve autonomic pathways, remain to be described.

7.3. ACUTE EMOTIONAL STRESS AND ET DURING MYOCARDIAL ISCHEMIA

All these observations have unclear repercussions for ischemia-induced VTs. This clinical setting is typical in the modern society, despite the potential causative relationship between acute emotional stress and coronary artery disease. The elevated prevalence of VTs among patients with implanted defibrillators following the terrorist attack on the World Trade Center in 2001 serves as an example (perhaps an extreme case) **(189)**. Furthermore, a cohort study **(204)**, examining patients with acute coronary syndrome after intense stress, reported significantly increased endothelin (and inflammatory markers, such as monocyte chemoattractant protein-1) in comparison to either a reference group or to healthy subjects. When taken together, the available evidence points to a pathophysiologic role of the endothelin system in acute coronary syndromes linked to emotional stress, but the potential effects on early-phase arrhythmogenesis call for more research **(217)**.

B. PURPOSE OF THE STUDY

This thesis aims to provide further insights into the pathophysiology of ischemia-induced ventricular tachyarrhythmias, focusing on the role of ET-1 in the activation of the central sympathetic system. Research will be extended to investigate acute coronary occlusion in the context of acute emotional stress. More specifically, **autonomic impulses and arrhythmogenesis during acute emotional stress will be investigated**: activation of the central sympathetic system can have important effects on cardiac electrophysiology, thus contributing to arrhythmogenesis. Moreover, **the role of endothelin-1 and ETB receptor in central sympathetic responses and the heart will be assessed**: ET-1 can modulate central sympathetic responses during acute myocardial ischemia. Furthermore**, insights on the effects of acute emotional stress on central sympathetic responses during myocardial ischemia will be provided**: Excessive sympathetic responses may occur as a result of acute coronary occlusion in the setting of acute emotional stress, mediated by ET-1. This effect may lead to enhanced arrhythmogenesis during ischemia. Understanding the pathophysiology of ischemia-induced arrhythmogenesis aims to reduce rates of sudden cardiac death, while revealing autonomic responses of this phenomenon could contribute significantly to understanding the pathogenesis of stress-related cardiovascular diseases. The knowledge that will be collected may form the basis for the creation of new therapeutic approaches in patients with myocardial ischemia who experience tachyarrhythmias and are often driven to sudden cardiac death. The outcomes of this thesis are likely to promote research in the field of post-infarction ventricular arrhythmias treatment.

C. SPECIFIC PART

8. MATERIALS AND METHODS

8.1. SELECTION OF THE ANIMAL SPECIES

This study conforms to appropriate regulations and guidelines. All procedures were conducted in accordance with the European Union (Protection of Animals Used for Scientific Purposes) Legislation 2010/63/EU **(218)**, approved by institutional ethical review committees (Pharmacology Medical School National and Kapodistrian University of Athens Animal Welfare and Ethical Review Board and Regional Municipality of Attika Welfare and Ethical Review Board) and conducted under the authority of the Project License (E01RWI01ΘK20210107, 574219/05-08-2020), respectively. All the experiments were also conducted according to the ARRIVE guidelines 2.0 **(219)**.

The species of experimental animal we chose to test the effect of acute emotional stress, as well as the effect of endothelin on the electrophysiological properties of the heart after acute myocardial infarction, is the Wistar rat. All animals were housed under optimal conditions in terms of temperature (20°C-22°C), humidity (70%), and light/dark cycles (12/12 hours) and had free access to food and water.

Experimental coronary occlusion was initially studied in large animals, such as the dog, the pig or the sheep. This is due to the fact that the heart anatomy of these animals closely resembles that of humans. Despite this fact, studies in the fields of physiology, pathology or pharmacology of the coronary circulation need a significant number of experimental animals in order for statistically significant differences to become apparent.

The use of large animals has a large financial cost, both for their acquisition or reproduction and for their accommodation in the experimental laboratory. In addition, the techniques used to induce myocardial infarction are more time-consuming and difficult and require the use of many

and expensive materials for their processing. Thus, experimental cardiology switched from large animal models to smaller ones, such as the rat and the mouse. The rat is bred and maintained in our laboratory facilities at low cost.

The early post myocardial infarction period in rats exhibits two distinct time periods, which are characterized by a high rate of ventricular tachycardia and fibrillation episodes, as in humans. **(83)**. In addition to these electrophysiological similarities, rats provide a reliable and reproducible model, with little variation in induced infarct size. For example, although dog studies have contributed to basic research, this model exhibits significant differences in coronary anatomy and collateral circulation size, resulting in substantial differences in transmurality and infarct size **(220)**. In contrast, in rats, a large transmural infarct is induced in a stable and reproducible manner due to the lack of significant collateral circulation. Even small differences in collateral circulation cause significant differences in arrhythmogenesis, exerting a greater influence than the size of the ischemic area itself **(220)**.

Another factor that makes the choice of rats ideal, is the fact that they frequently develop ventricular arrhythmias during ischemia, but without these being highly lethal **(83)**. This phenomenon has been linked to the heart's small size and electrophysiological properties, the age of the animal and the state of the autonomic nervous system **(221)**. Thus, it is possible to study many episodes of ventricular arrhythmias of a small number of experimental animals, since these episodes self-terminate up to 97% **(83)**.

Concerning the acute emotional stress protocol, animal models of intense unpredictable stress are broadly utilized and contribute to investigating various viewpoints of the underlying physiology and pathophysiology. Restriction **(222)** and air-jet stress **(223)** in rats have risen as simple and effective translational models of acute emotional stress. These methods, alone or in combination, have been used for decades, and their impact on sinus heart rate (HR) has been described in detail.

The strain of the rat also plays an important role. Sprague-Dawley rats show higher infarct size and mortality rate, compared to Wistar rats, resulting in a significant limitation of the study of

the second post infarct arrhythmogenic phase, as well as a larger requirement of experimental animals' number **(224)**.

More choice criteria in the planning of our study were the rat's sex and age. To overcome possible sex-related differences, given the previously reported differences in HR responses to stress, all rats were male **(225)**. The rats tested were 18 to 20 weeks of age (weighing 374±57 g), since it is indicated that this age range provides beneficial experimental conditions, not only in terms of heart dimensions, but also in terms of perioperative mortality **(126, 128, 226)**.

To study the effect of endothelin on the electrophysiological properties of the heart, the endothelin receptor B (ETB) deficient Wistar-Imamichi rat model was used. This model has already been characterized **(32, 227)** and a colony was mercifully provided to our animal facility by Professor M. Yanagisawa (University of Texas Southwestern Medical Center, Dallas, TX, USA). In this rat strain, completely non-functional ETB receptors are expressed as a result of a 301-bp deletion in ETB receptor gene that leads to abnormal mRNA transcript **(10)**. The absence of functional ETB receptors in this strain has been confirmed by in situ hybridization for ETB mRNA as well as by polymerase chain reaction **(32)**. Dopamine β- hydroxylase promoter has been utilized to direct ETB transgene expression and to support typical enteric nervous system development, to avoid premature death of intestinal obstruction in these rats **(227)**. As a result, these rats live into adulthood, but lack ETB receptors in the cardiovascular system, making them a valuable tool in the study of the pathophysiology of ET-1. Their deficiency results in ten-fold elevated levels of immunoreactive circulating plasma endothelin-1 **(32)**. Moreover, higher ETA receptor protein expression has been found in homozygous, compared to heterozygous rats of this strain **(228)**. As noted above, dopamine β-hydroxylase promoter control is absent within the cardiovascular system of this rat **(32, 227)**; thus, this phenotype permits precise evaluation of ETB receptors in experimental models of myocardial ischemia.

8.2. ANATOMY OF THE CORONARY VESSELS IN THE RAT

Immediately after emerging from the root of the aorta, the two coronary arteries enter the myocardium, which surrounds them throughout their entire course. Hence, as well as for the fact that they have a small size they are difficult, if not impossible, to be seen, even with the use of a microscope.

For this reason, we use specific anatomical guide points for the ligation of the left coronary artery. Specifically, the latter origins from the aorta and between the left side of the pulmonary conus and the left atrium **(229)**. Thus, the ligation of the left coronary artery at a point close to its origin is achieved by inserting a needle into the pulmonary conus wall which exits near the auricle of the left atrium. The coronary circulation is mostly carried out by one main branch, which divides near the apex of the heart. There are also several small branches arising from the left coronary artery, but they are not significant **(230)**.

Likewise, the right coronary artery originates from the root of the aorta and runs between the right side of the pulmonary conus and the right pulmonary auricle **(229)**.

8.3. EXPERIMENTAL MODEL

The experimental study was conducted on 48 male Wistar rats and 48 male ETB deficient rats, aged 18-20 weeks and weighing 374±57 g. The animals received humane care and every effort was made to minimize their suffering.

To safely draw conclusions from the effect of acute emotional stress, as well as the effect of endothelin, or the results of the combination of acute stress and elevated plasma endothelin levels, on the electrophysiological properties of the heart before and after acute myocardial infarction, the study was conducted in two parts.

A) In the *first part*, the effects of acute emotional stress on the heart rate variability, sympathetic and parasympathetic indexes, blood pressure and arrhythmias in both Wistar and ETB deficient rats, were studied (Fig.14).

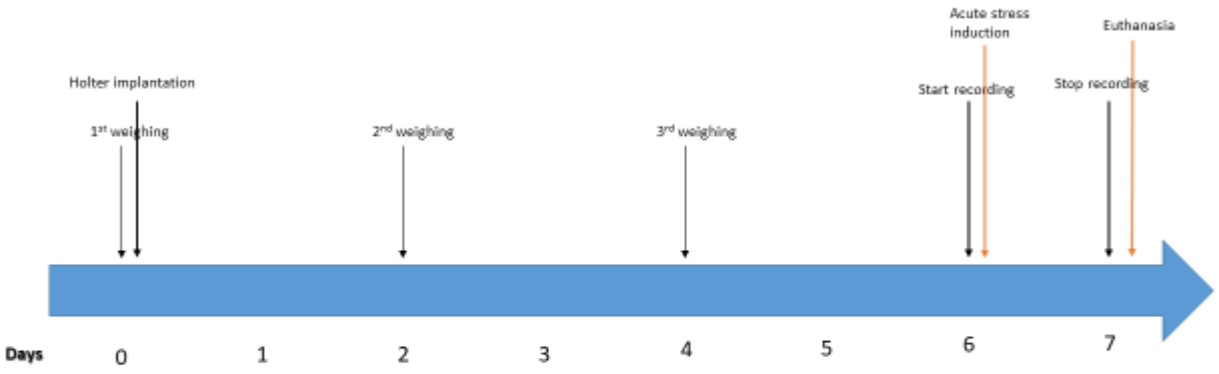


Figure 14: *First part of the study: Stress protocol. Control groups experienced the same treatment without the acute emotional stress.*

B) In the *second part* of the study, the results of myocardial infarction, in combination with acute stress, on the autonomous activation and arrhythmogenesis after myocardial infarction, were analyzed in both Wistar and ETB deficient rats (Fig.15).

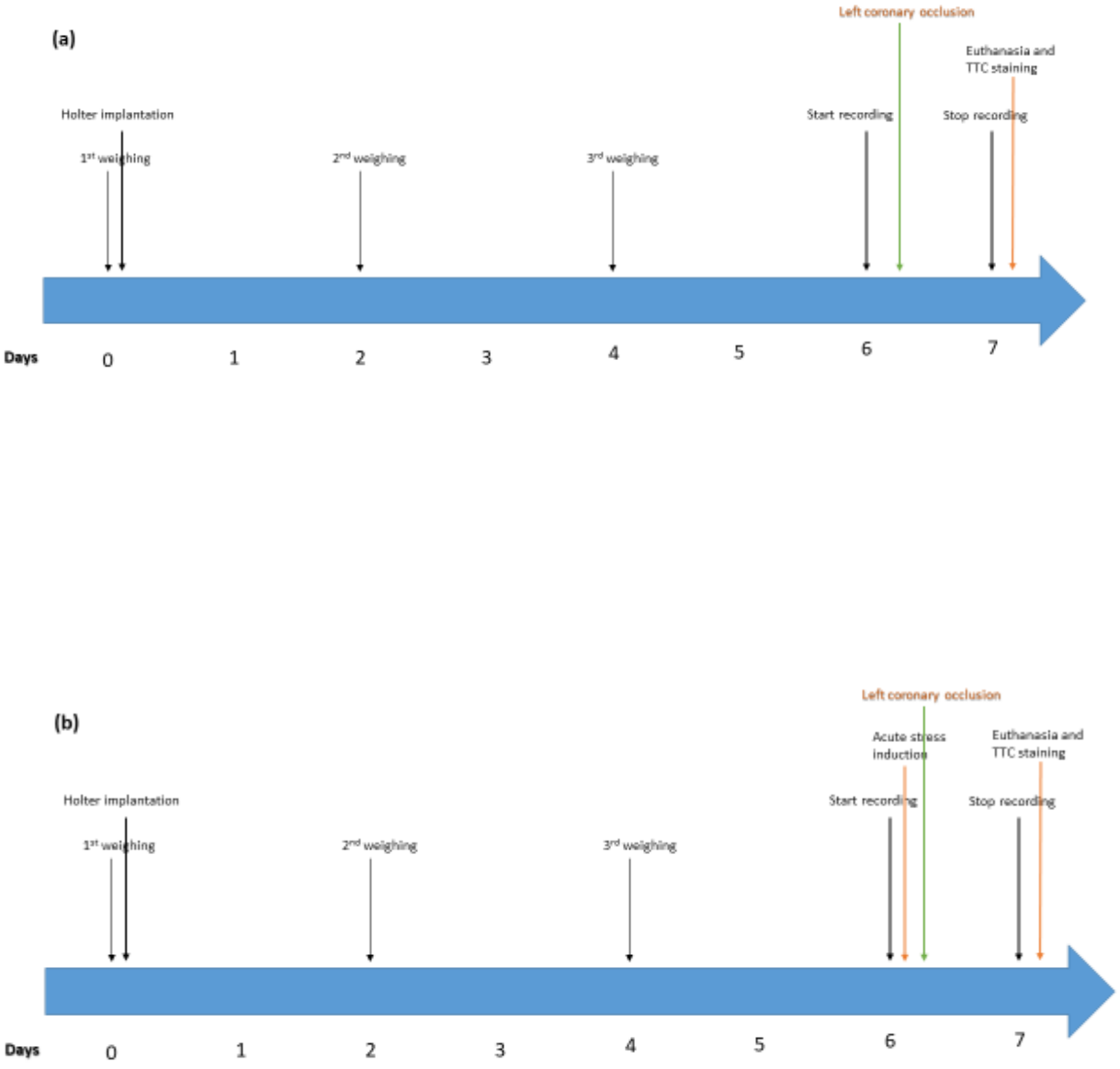


Figure 15: *Second part of the study: Stress and myocardial infarction protocol. (a) Control groups had the coronary occlusion surgery. (b) The other groups experienced acute stress before the ligation of the left coronary artery.*

8.3.1. Blood pressure measurement

Blood pressure was measured in 8 Wistar and 8 ET-B deficient rats during morning hours, while absolute silence prevailed. To measure the pressure, it is necessary to place the rat in a restrainer. Thus, the blood pressure measurements occurred before (baseline period), during the air-jet stress (stress period) and after the termination of the stress protocol (recovery period). The rat was placed in the restrainer, and covered with a towel, so that it remained calm and its temperature maintained. The blood pressure was measured non-invasively from the tail, with the use of a pulse transducer and a tail cuff. Both the pulse sensor and the tail cuff connect to the Non-invasive Blood Pressure System for mouse or rat (NIBP System, ADInstruments Inc., Colorado Springs USA) (Fig.16). This reliable system allows to measure blood pressure without operating the animal, avoiding unnecessary pain and time loss **(231)**. The specialized tail cuff and pulse transducer, are used for intermittent rat blood pressure measurement based on the periodic occlusion of tail blood flow. Once the blood flow stops, the tail sensor records and transmits the pulse signal to the central system. The central system was set to transmit in a pulse range of 90-420 BPM. The NIBP System outputs the pressure and pulse signals to PowerLab via a BNC connection.

Figure 16: (*Left) Tail cuff, (Right) Non-invasive Blood Pressure System for mouse or rat (NIBP System, ADInstruments Inc.)*

Two channels were chosen, one presented the pulse signal from the tail sensor and the second showed the intensity of the pressure (Fig.17). Due to the increased pressure exerted by the tail cuff on the tail, the blood flow stops and until it resumes we cannot see the pulse. As soon as the tail cuff was slightly relaxed, the pulse appeared, and at this point we spotted the systolic blood pressure. Regarding the diastolic blood pressure, its value was spotted at the point where the first normal pulse peak appeared. However, for this method to be considered reliable we repeated each measurement at least 10 times and final systolic and diastolic blood pressure values are presented as mean ± standard deviation of the 10 measurements **(232)**.

Figure 17: *Rats pulse during recording. In the upper channel the intensity of the blood pressure is presented in mmHg. In the second channel the normal heart pulse is presented.*

8.3.2. Anesthesia – Analgesia

The rats were placed in an induction cage with 5%-isoflurane (Abbott Laboratories, Abbott Park, IL, USA) for 1-2 minutes. Therefore, they were anesthetized for the intubation to occur. The rats were intubated under laryngoscopy by inserting a 18G venous catheter connected

to a small animal mechanical ventilator (Model 7025, Ugo Basile, Verona, Italy) at 85 breaths/min and tidal volume 2,5 ml; anesthesia was maintained with a mixture of oxygen and 2.5% isoflurane, a regimen with rapid post-anesthesia recovery, effective myorelaxant and analgesic effects **(233, 234)**. Moreover, the experimental model of chronic myocardial infarction in rats, involving lateral thoracotomy, intubation, and administration of a mixture of oxygen and isoflurane is associated with the least excitatory mortality. In addition, evidence suggests that isoflurane appears to be devoid of anti- or proarrhythmic activity during the first hours of ischemia and infarction **(235)**. Buprenorphine was subcutaneously administrated for analgesia, pre-operatively (0.005 mg/100 g) **(236)**.

8.3.3. Implantation of Telemetry Transmitter (Holter)

Miniature telemetry transmitters (TCA-F40, Data Sciences International, DSI, Arden Hills, MN, USA) were placed in all rats. This is a monitoring system consisting of a transmitter, implanted in the animal's abdominal cavity, capable of transmitting an electrocardiographic signal continuously, and a plate – receiver (RPC-1, Data Sciences International), which is placed under the rat's cage. This wireless heart rate monitor allows for long-term recording of ECG signals, without the effect of anesthesia, which affects the appearance of ventricular arrhythmias after myocardial infarction through its effect on potassium concentrations (increase) and on the autonomic nervous system **(237, 238)**. The implanted transmitter continuously sends an ECG signal to the plate – receiver. These data are then transferred and stored to the computer's memory card, being ready to process.

The implantation surgery begins when the animal is properly anesthetized and the analgesic is injected. An incision is made in the middle of the abdomen, the peritoneal cavity is opened and a small transmitter (T-F40 Data Sciences International) is placed in it. The telemetry transmitter weighs 7 gr and has a volume of 3 cm^3. This transmitter has two stainless steel cables, which are covered with silicone along their entire length, except for the 1 final cm. These two ends serve as the electrodes of the system. Thus, these are led subcutaneously

and secured under the right axillary and the left hind-limb area, representing ECG lead II **(239)**. The transmitter body is attached to the abdominal wall along the incision line with 4-0 proline suture as the incision is closed (Fig.18). Rats were then allowed to recover from surgery in their home cages for at least 5 days before experiments began. On the day of the experiment the rat is placed in an individual cage located above the special plate - receiver (RPC-1, Data Sciences International).

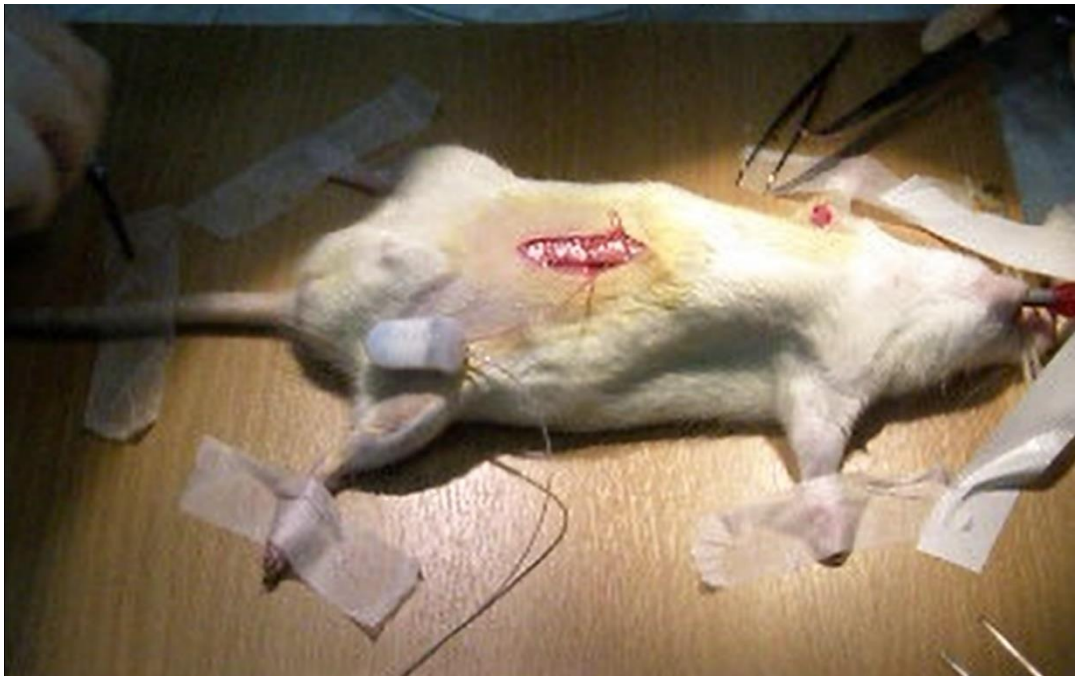


Figure 18: *Implantation of a telemetry transmitter in the peritoneal cavity of the intubated and anesthetized rat.*

The received ECG signal is then amplified and digitized. After digital-to-analog conversion and filtering at 100 Hz (Data Exchange Matrix, Data Sciences International), a continuous stream of data is provided to the computer. Using the software Dataquest Acquisition & Analysis System ART 2.2 (Data Sciences International), the digitization is done with 16-bit precision and the ECG can be represented in real time with 500 Hz sampling, while at the same time the data is stored for further analysis. ECG recording for each rat lasted 24 (for the control rats) to 28 hours, starting 20 minutes before any of the experimental procedures, acute stress or left coronary artery ligation, and ending 24 hours after.

8.3.4. Acute stress

On the day of stress induction, the cages (containing rats with implanted transmitters) were placed on top of telemetry receivers (RPC-1, Data Sciences International), through which the ECG signal was recorded by the acquisition software (ART 2.2, Data Sciences International). The room was kept quiet, maintaining the light/dark cycle, with all experiments performed during the morning hours. The stress protocol used in the experiments (Fig.19), adopted from previous descriptions, consisted of restraint **(222)** followed by air-jet stress (AJS) **(223)**, with a total duration of 43 minutes.

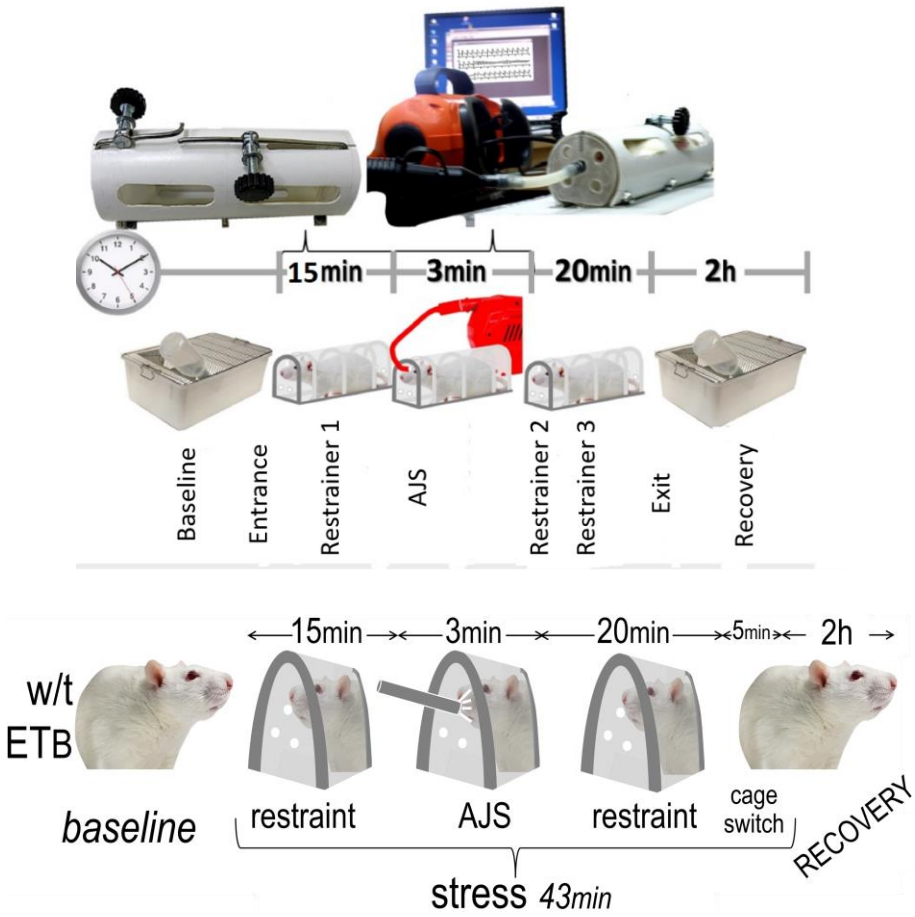


Figure 19: *The stress protocol included the successive use of restraint and air-jet stress (AJS) and cage-switch. Autonomic variables were evaluated at eight time frames, each of five-minute duration (upper figure). (Modified from reference* ***(198)*** *With permission). The four stages of the stress protocol (lower figure).*

The rats were placed in a tubular custom-made restrainer (inner diameter: 8.25cm) with sufficient ventilation. Fifteen minutes thereafter, air-jet stress was initiated for three minutes, consisting of 18 pulses (each of two-second duration, delivered at eight-second intervals). Specifically, air was blown (at 10 L/min) **(240)** to the forehead by a pump (BDINF20C, Black & Decker, Towson, MD, USA) with its outlet attached to a 3.2 mm opening at the front of the restrainer (Fig.20). Restraint was sustained for further 20 minutes, after which period the rats were returned to their cages, under continuous ECG monitoring. The following five-minute periods were analyzed: baseline, restraint onset, restrainer-1 (12min following the onset of the previous period), air-jet (consisting of 18 air-pulses, each of 2s duration, given at 8s intervals via air pump at 10L/min), restrainer-2 (immediately after the termination of the previous period), restrainer-3 (the last five minutes in the restrainer) and exit (the first five minutes in the cage). We included the latter in the period of AES, as it encompasses cage-switch, an established aversive stimulus **(241)**, reiterated by our groups recent experience **(198)**. Findings from the six stress-periods are reported either separately or averaged under a single period. In addition, two five-minute periods of recovery were analyzed, namely recovery-1 (commencing five minutes after return to the cage) and recovery-2 (the last five minutes of recovery), reported either separately or as a single average.

Figure 20: *Rat in tubular restrainer during the induction of acute Air Jet Stress protocol.*

8.3.5. Left coronary artery ligation

After anesthesia and intubation, rats were placed in a supine position on a temperature control pad. Left lateral thoracotomy was performed and pectoral region muscles were separated, exposing the ribcage. If injury to the internal mammary artery occurred, bleeding was gently stopped by applying pressure. Using curved forceps, the intercostal muscles (between the 5th and 6th ribs), approximately 2 mm to the left of the sternum, were transected, so as to avoid bleeding from the internal epigastric artery. The created gap was expanded with the curved forceps. To maintain the opening, a thoracic retractor was used.

The pericardium was separated with the use of blunted forceps. A 6-0 propylene monofilament suture with round bodied needle and 3/8 circle, was placed over the apex of the heart to immobilize it in the thoracic cavity. A 5-0 propylene monofilament suture with round bodied needle and 3/8 circle, was inserted from the pulmonary cone to the left atrial appendage. Thereafter, the suture was tightened to ligate the left coronary artery 4 mm from its origin **(242)**. Thus, the left coronary artery was occluded near its exit, causing a large infarct (Fig.21). The ligation of the left coronary artery following these anatomical landmarks, ensures high repeatability of the method and comparable infarct size **(126, 243)**.

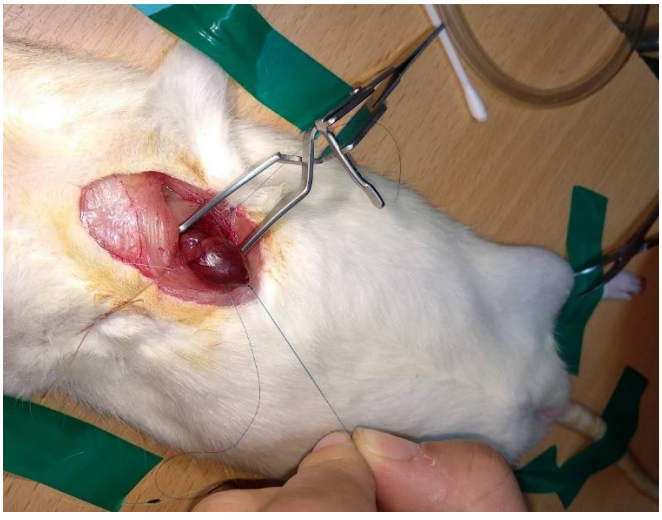


Figure 21*: Restraint of the heart through the apex suture and left coronary artery ligation in the rat.*

The incision was then sutured in three layers and gentle bilateral pressure was applied to evacuate air from the thorax'. Thanks to the skin, subcutaneous tissue, and muscles functioning as a valve, the pressure helps prevent pneumothorax. After the induction of acute myocardial infarction in rats, changes are observed both in terms of the functionality of the left ventricle and in its morphology. Myocardial infarction was confirmed, immediately after the occlusion of the left coronary artery, by the inspection of decreased mobility of the left ventricle as well as a change in the area color. Moreover, the presence of ST-segment elevation in two, or more, leads was evidence of an acute myocardial infarction. When surgery was completed, isoflurane was discontinued and rats regained consciousness within minutes.

8.3.6. Arrhythmia analysis

The stored ECG signals were analyzed according to the guidelines of the 'Lambeth Conventions' **(224, 244)**. Premature ventricular contractions (PVCs), defined as single discrete complete electrical events in the ventricle, couplets (two consecutive PVCs) and triplets (three consecutive PVCs) were counted. Ventricular tachycardia (VT) was defined as the appearance of 4 or more consecutive premature electrical complexes (PVC); early QRS complexes in relative to the P wave (Fig.22). Ventricular fibrillation (VF) was defined as the appearance of a signal that changes from cycle to cycle in terms of frequency and morphology, or a signal in which the various QRS complexes cannot be distinguished from each other. Even with these criteria, it is often difficult to distinguish VTs from VFs, considering that many times the two arrhythmias can pass into one another, without a clear boundary between them **(83, 244)**. Thus, the sum of VT+VF episodes was calculated for each rat. The number and duration of bradyarrhythmic events were also recorded, including sinus pauses and atrio-ventricular blocks (AV block). Sinus pauses appear when the sinus node alters conduction to the atrial myocardium. This disorder is often intermittent, resulting in the absence of atrial contraction, for an integer multiple of the baseline interval. On the other

hand, an AV block is a partial or complete interruption of impulse transmission from the atria to the ventricles **(245)**. The duration of each episode was determined using the graded scale provided by the software.

In the experimental rat model of infarction, the incidence of ventricular arrhythmias during the first twenty-four hours after acute myocardial infarction is not uniformly distributed. Specifically, they appear to have two periods characterized by high arrhythmogenic activity. The first one consists of the first hour after the occlusion, followed by a period of low activity. The second period is determined between 1.30 to 9.00 hours after the occlusion. These two phases appear to be caused by different mechanisms. First-phase arrhythmias are thought to be caused by reentrant mechanisms, while second-phase arrhythmias appear more likely due to increased automaticity **(83)**.

To simplify the results and easier understand the role of the endothelin and acute emotional stress post-MI, the analysis performed in this study was not done for the two distinct, strongly arrhythmogenic periods following coronary occlusion, but it was performed as they were one period. For the rat groups that had MI, the analysis was performed for an eight-hour post-myocardial infarction period. Thus, the number of episodes or their duration are expressed as a sum of episodes recorded in the eight-hour observational post-MI period.

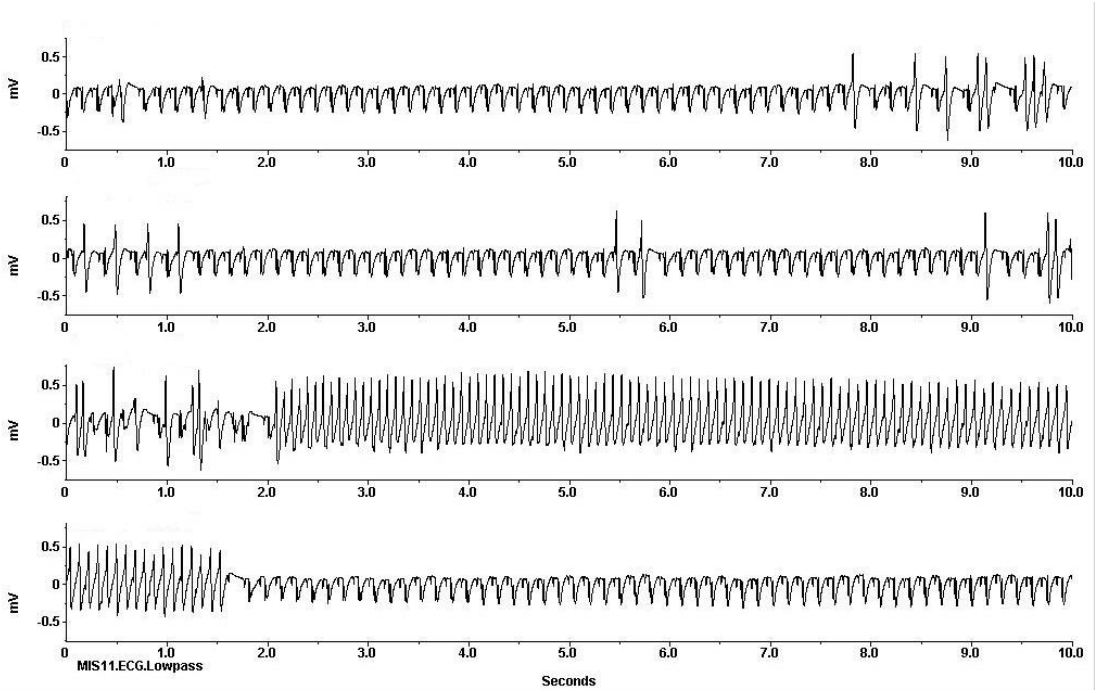


Figure 22*: Example of PVCs, couplets, triplets and ventricular tachycardia.*

8.3.7. Heart Rate Variability analysis

With the use of the software (Dataquest Acquisition & Analysis System ART 2.2, Data Sciences International), R spikes and then R-R intervals were identified in each stored data series. Given specific constraints, the program algorithm excluded non-sinusoidal pulses and averaged the R-R interval for 5-min time steps. The typical cut-off frequency ranges for low-frequency (LF) and high-frequency (HF) powers (LF, 0.5–0.8 Hz; HF, 0.8–2.4 Hz) seemed a good compromise to gauge the sympathetic and parasympathetic components of heart rate variability (HRV). HRV analysis was performed from consecutive inter-beat intervals, with the use of the Kubios software (University of Eastern Finland, Kuopio, Finland); indices at each period were averaged from five-minute recording intervals. As no single HRV index provides accurate description of the activation of each autonomic arm, we used those derived from a combination of variables, as previously outlined **(246)**. The sympathetic nervous system index (SNSi), which represents the sympathetic nervous system activity compared to normal values,

was computed from three variables, namely (a) mean HR, (b) Baevsky's stress index, and (c) the length of the distribution of Poincaré plots after nonlinear analysis (SD2). The parasympathetic nervous system index (PNSi), which represents the parasympathetic nervous system activity compared to normal values, was computed also from three variables, namely (a) the mean inter-beat interval (RR), (b) the root mean square of successive differences between inter-beat intervals in time-domain analysis (RMSSD), and (c) the width of the distribution of Poincaré plots (SD1). Individual variability in SNSi and PNSi responses was accounted for by their expression as percent change from baseline values.

As mentioned before, acute emotional stress triggers autonomic responses that affect sympathovagal balance and different pathophysiological mechanisms are responsible for the two arrhythmogenic periods following coronary occlusion. However, the temporal pattern of changes in each autonomic arm during stress, MI and recovery remains unclear. Therefore, in this study, sympathetic and vagal activity are separately analyzed for the different stages of those events.

8.3.8. Activity analysis

Voluntary motor-activity was recorded with the use of the analysis software (ART, Transoma) for the baseline, the two-hour recovery periods post-MI and/or post-stress (Recovery 1 and Recovery 2). The rats' activity was assessed by the number of counts, generated by strength variations in the telemetry-signal, relative to the animal's location in the cage; these counts correlate with the incidence and severity of acute left ventricular (LV) failure **(247)**. Voluntary physical activity also provides a measure of continuing anxiety and is used as a marker of post-stress adaptation **(248)**. To account for variability at baseline, the percent change during recovery is reported.

8.3.9. Determination of the infarct size and euthanasia

The technique used for calculating infarct size is the 2,3,5-triphenyltetrazolium chloride (TTC) staining (2,3,5-Triphenyltetrazolium chloride, T8877, Sigma-Aldrich). This technique is used to differentiate metabolically active and inactive tissues and is based on the ability of succinate dehydrogenase enzyme to react with tetrazolium salts and form a red-tinged surface. Hence, the myocardium that has survived after coronary artery occlusion is stained red, while necrotic areas appear white (Fig.23).

TTC method is capable of detecting myocardial necrosis at least 3 hours after the onset of ischemia in rats. Experimental animals were subjected to this procedure 24 hours after the coronary occlusion **(249)**. Rats were anesthetized with isoflurane and cervical dislocation was performed to euthanize them. A careful left thoracotomy was made to expose the heart, so as not to injure any intrathoracic organ. The heart was perfused from the left ventricle with 1X PBS with 0,2 M KCl, not only to remove the blood but also for the heart's mechanical function to be interrupted and cause dilation **(250)**. The myocardium was then quickly excised and washed with 1X PBS. After being cleaned, it was placed in a freezer at -20°C for 1 to 2 hours. The heart was then cut into approximately 4 to 5 parallel slices, which were immersed in the TTC solution for 15 to 20 minutes at 37°C. Areas that had not died were thus colored bright red. When this procedure was completed, the slices were immersed in a 10% formalin solution for about 20 minutes. In this way the staining was fixed and the contrast between the surviving red tissue and the dead white became obvious. Then the slices were placed between two glass slides, which have a fixed distance of 2 mm between them and a high-resolution scan, with a simultaneous scan of a sub-decameter for grading, followed. With the use of the software ImageJ Fiji 1.46, the surface of the infarcted and non-infarcted areas were measured. The fraction of the infarcted to total area of each slice was multiplied by the weight of each slice to calculate the infarct mass. The sum of the infarct masses was divided by the total left ventricular mass, thus determining the infarct size **(251)**. Transmitters were removed from the peritoneal cavity, to reuse.

Figure 23*: Sections of the ventricular myocardium stained with TTC. In the image above, the infarcted area (pale zone) is clearly visible.*

8.4. STATISTICAL ANALYSIS

Values are reported as mean ± one standard deviation. Baseline variables in the two rat-strains and voluntary activity post-stress in the two groups were compared with t-test. Changes over time were assessed with the use of (two-way) analysis of variance for repeated measures, with rat-strain and time as between- and within-groups factors, respectively. Baseline values of all variables in both ETB groups and both control groups, of each part of the study, were similar and are presented as the respective average. Differences (between- and within-groups) at each prespecified time-period were evaluated with the use of post-hoc Tukey's HSD test. Variables describing brady- and tachyarrhythmias were not normally distributed, according to the (Lilliefors corrected) Kolomogorov-Smirnov test **(252)** and were compared with non-parametric tests, namely Mann-Whitney U-test or Kruskal-Wallis analysis of variance, as appropriate. Statistical significance set at an alpha level of 0.05.

9. RESULTS

9.1. FIRST PART RESULTS

This part of the experimental study included 28 male Wistar (w/t) rats and 28 male ETB deficient rats, aged 18-20 weeks and weighing 374±57 g. Eight Wistar and eight ETB deficient rats were used to measure the blood pressure, while the rest of them took part in the evaluation of the heart rate variability and arrhythmias. Each experimental group of the evaluation of the heart rate variability and arrhythmias included 10 rats. The average weight of the Wistar rats was 436±48 g, while the average weight of the ETB deficient rats was 276±41 g. For the two different genotypes, after randomization, a stress group and a control group (no stress) where formatted.

9.1.1. Mortality

During the three-hour observation period, no experimental animals (0/56, 0%) died.

9.1.2. Baseline differences between the two rat strains

Table 5 demonstrates the baseline characteristics in wild-type and ETB-deficient rats. Voluntary activity and HR were comparable between groups, as were time-domain HRV variables. By contrast, the ratio of low- (LF) to high-frequency (HF) spectra in the frequency-domain analysis, depicting steady-state autonomic balance, indicated vagal dominance in ETB-deficient rats. This finding reflected differences in both autonomic arms, as shown by lower LF and higher HF values, indicating lower sympathetic and higher vagal activity, respectively. Lower sympathetic activity in ETB-deficient rats was evident also by SNSi, whereas the difference in the vagal index PNSi was of marginal statistical significance.

Differences in PVCs or tachyarrhythmias were absent at baseline. However, occasional bradyarrhythmic episodes in the form of sinus pauses were recorded in ETB-deficient, but not in wild-type rats (Fig. 24). No atrioventricular conduction disturbances were present in any group.

Table 5: *Baseline values for the wild-type and the ETB deficient rats. The table shows the mean and standard deviation of each parameter for each group in baseline. SD=standard deviation, HR=heart rate, LF=low frequency, HF=high frequency, SDNN=standard deviation of RR intervals, RMSSD=root mean square of successive differences between inter-beat intervals in time-domain analysis, SNS=sympathetic nervous system, PNS=parasympathetic nervous system, PVCs=premature ventricular contractions. Significant p values are displayed in red.*

Variables	wild-type	ET_B-deficient	*p*-value
	Mean ± SD		
Sympatho-vagal balance			
Mean HR (bpm)	314±53	337±34	0.1029
LF/HF	0.021±0.011	0.011±0.0007	**0.0005**
SDNN (ms)	4.122±4.638	5.309±4.144	0.3984
RMSSD (ms)	7.285±8.242	9.562±7.616	0.3700
Sympathetic activity			
Power LF	1.874±0.955	1.129±0.0697	**0.0012**
SNS index	451±212	325±75	**0.0172**
Vagal activity			
Power HF	97.82±1.02	98.66±0.08	**0.0007**
PNS index	-5.459±0.179	-5.547±0.106	0.0671
Bradyarrhythmias			
Sinus pauses	0	0.50±1.00	**0.0197**
AV block episodes	0	0	(-)
Tachyarrhythmias			
PVCs/h	0.250±0.55	0.35±0.87	0.8540
Couplets/h	0	0.10±0.44	0.3421
Triplets/h	0	0.15±0.36	0.0803
Voluntary activity			
Activity counts/hour	444±572	477±428	0.8346

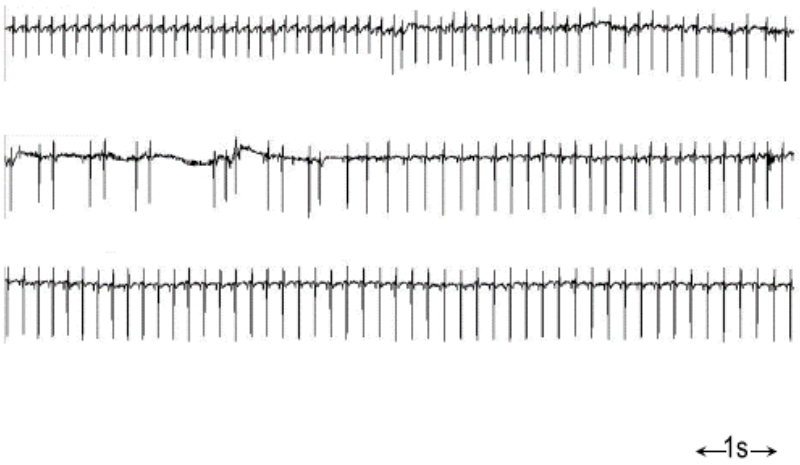


Figure 24: *Sinus pauses of the ETB rats during baseline recordings. Each row represents 10 seconds of recordings.*

9.1.3. Responses to acute emotional stress

Table 6 summarizes the changes that occur during acute emotional stress (AES) in wild-type/Wistar rats and ETB deficient rats. Specifically, HR increased during AES in both wild-type and ETB deficient rats, but only in wild-type rats the difference was statistically significant. Moreover, systolic BP displayed significant differences during stress and recovery in each group. While ETB deficient rats had no difference in their voluntary motion during the two-hour recovery period, voluntary motion of wild-type rats was more pronounced in the stress group than in the controls. Specifically, the total number of motion counts was higher ($p = 0.035$) in the former (8878 ± 1715) than in the latter group (2790 ± 1186).

Table 6: *Changes during stress for the wild-type and the ETB deficient rats. The table shows the mean and standard deviation of each parameter for each group in baseline and stress conditions.*

SD=standard deviation, SNS=sympathetic nervous system, PNS=parasympathetic nervous system. Significant p values are displayed in red.

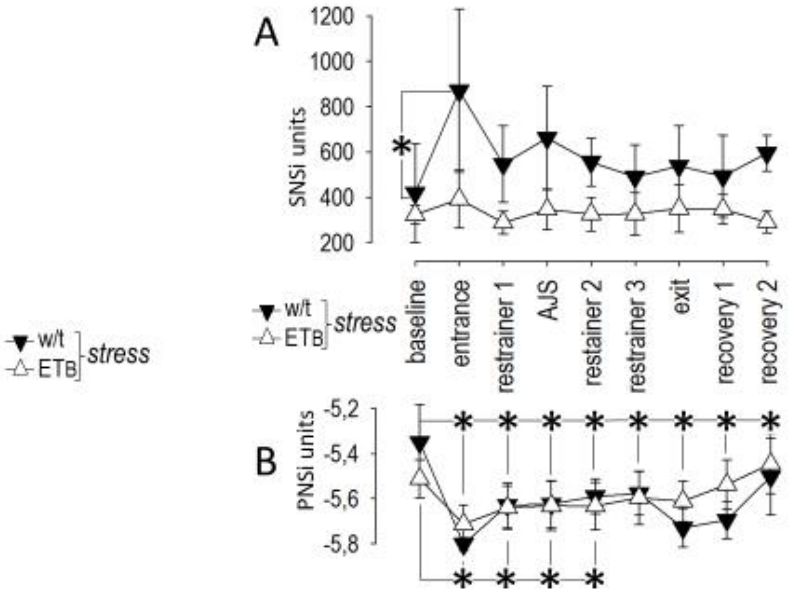
Variables	Baseline	Stress	*p*-value
	Mean ± SD		
Heart rate (bpm)			
wild-type	314 ± 53	338 ± 29	**0.0001**
ETB-deficient	337 ± 34	373 ± 38	0.9999
Systolic blood pressure (mmHg)			
wild-type	110 ± 11	128 ± 18	**0.0443**
ETB-deficient	115 ± 5	130 ± 12	**0.0439**
Sympathetic activity (SNSi units)			
wild-type	451 ± 212	611 ± 111	**0.006**
ETB-deficient	325 ± 75	339 ± 56	0.9000
Vagal activity (PNSi units)			
wild-type	-5.459 ± 0.179	-5.659 ± 0.071	**0.0001**
ETB-deficient	-5.547 ± 0.106	-5.638 ± 0.088	**0.0019**

Autonomic responses to stress

When examining more specifically the course of the autonomic branches during the protocol procedures it seems that the extreme activation of the SNSi, of the Wistar rats, occurs during the restriction of the rats in the restrainer (from 451.33 ± 212.39 to 869.90 ± 359.33, p = 0.0002) with a concurrent vagal withdrawal (from -5.459 ± 0.179 to -5.802 ± 0.030, p = 0.0001) (Graph 3). Following that, the SNSi reduces and during the air jet presents a small and statistically insignificant increase which tends to be eliminated over time, but still remains insignificantly elevated in comparison to baseline until the last experimental time frame (recovery 2: 595.23 ± 79.48, p = 0.5491). During all those periods, the temporal pattern of PNSi in this group displayed a marked decrease after the onset of acute stress, followed by low values during the remaining period of observation. As shown in Graph 3, ETBs during the entire period of stress and recovery had stable SNSi values, but the PNSi of those animals reduced significantly during the stress protocol; of note from -5.547 ± 0.106 to -5.713 ± 0.086 with p = 0.0001 during the entrance of

the animals to the restrainer and to -5.635 ± 0.103 with p = 0.0036 during the rest time frames of stress (mean value of the three remaining periods with asterisks). Over time, the PNSi of the ETBs returned back to baseline values.

In summary, compared to baseline, SNSi increased sharply from baseline in wild-type rats at the onset of stress, with subsequent decline during the remaining period of stress and during recovery (Fig. 3A). Changes in PNSi were more prolonged in this group; specifically, PNSi decreased from baseline at the onset of stress and remained low during the period of stress and during recovery (Fig. 3B). Contrasting the response observed in wild-type rats, SNSi remained unchanged from baseline during stress and during recovery in ETB-deficient rats (Fig. 3A). As in wild-type rats, more prolonged PNSi changes were observed in this group, although this variable returned to baseline values earlier, i.e., at the end of AES and prior to the onset of recovery (Fig. 3B).

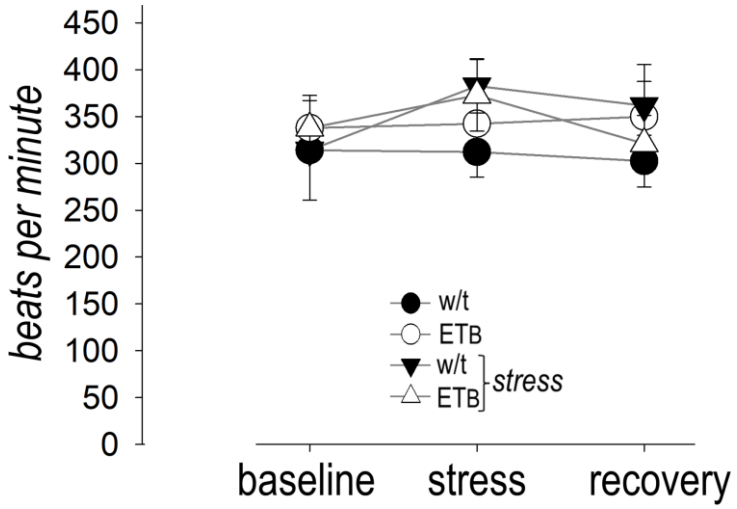


Graph 3: *Sympathetic (A) and Vagal (B) responses during and post-stress. The sympathetic nervous system index was significantly elevated in the beginning of the stress protocol in the Wistar group (asterisk) and returned to normal values post-stress, while the parasympathetic nervous system index was significantly lowered in this group during the entire period of stress and*

recovery (asterisks). On the contrary, the ETBs during the entire period of stress and recovery had stable SNSi values and the PNSi was significantly reduced during stress (asterisks) and returned back to normal during recovery. Asterisks indicate p-value < 0.001.

9.1.4. Heart rate

The two stress groups (Wistar stress and ETB stress) had higher heart rate during the stress period in comparison to the non-stress groups and the baseline values, although the difference was not significant in the ETB stress group. The heart rate of the Wistar rats rose from 285 ± 47 bpm to 383 ± 29 bpm during the stress period (p = 0.0001) and as it appears from the recovery values, in both the stress groups, has a tendency to return to normal over time. In general, there was no difference in the heart rate between the four groups, during all the three time frames (baseline, stress period and recovery period). Heart rate values are shown in Graph 3 and Table 5.



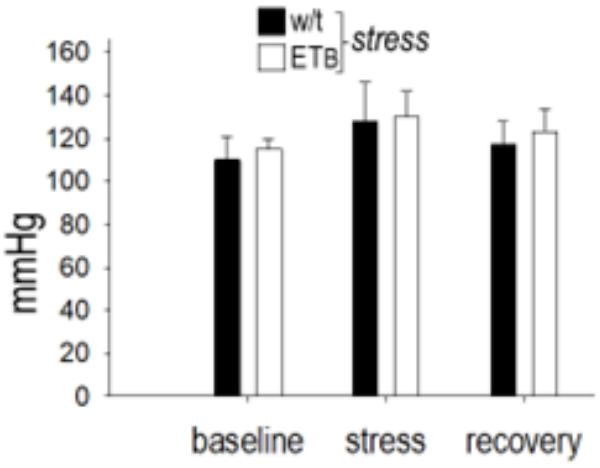
Graph 4: *Heart rate of the four groups. No statistical difference was found during all the three time frames.*

Table 7: *Heart rate recording. The table shows the mean and standard deviation for each group in each time frame. (SD=standard deviation).*

	MEAN HR (BPM) ± SD			
	Wistar	*ETB*	*Wistar stress*	*ETB stress*
BASELINE	343 ± 44	350 ± 37	285 ± 47	325 ± 29
STRESS	312 ± 27	342 ± 37	383 ± 29	373 ± 38
RECOVERY	303 ± 28	350 ± 38	362 ± 44	321 ± 30

9.1.5. Blood pressure

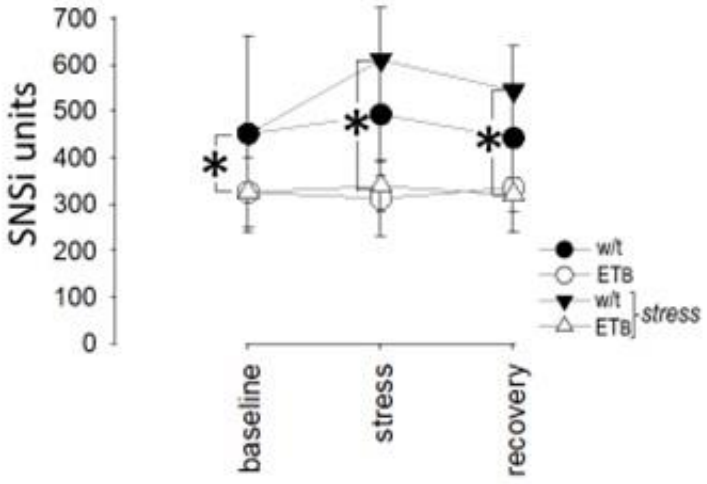
Systolic BP displayed significant differences during stress and recovery in each group, without differences between them. In Wistar rats, systolic BP (expressed in mmHg) rose from 110 ± 11 (baseline) to 128 ± 18, during stress. In ETB-deficient rats, a rise was also present, with respective values recorded at 115 ± 5 and 130 ± 12. During recovery (i.e., at the 5th min post-stress), systolic BP returned to baseline values, 117 ± 11 in Wistar rats, and at 123 ± 11 in ETB-deficient rats. The observed differences between groups failed to reach statistical significance (Graph 4).



Graph 5: *Systolic blood pressure. No statistical difference was found during all the three time frames.*

9.1.6. Sympathetic activity

SNS index was comparable between the two groups of Wistar rats at baseline, as it also was for the two ETB groups. When comparing the two genotypes at baseline, though, SNSi of the Wistar rats is significantly higher than the ETB rats ($p = 0.0491$). The SNSi during stress and recovery (expressed in SNSi units) is shown in Graph 5; SNSi remained stable in controls and the ETB stress group over time ($p = 0.9$), but a significant variance was present in the stress group of the Wistar rats; of note, the SNS index was significantly elevated to 610.64 ± 111.18 during stress, from 419.73 ± 217.89 in baseline ($p = 0.006$). Between-group comparisons for the Wistar and the ETB rats revealed significantly higher SNSi in the stress group of the Wistar (than the ETB) during the entire period of stress and at the recovery (Table 6). From Graph 5, it appears that there is a tendency of the SNSi to return to normal values post-stress.



Graph 6: *Sympathetic nervous system response. Sympathetic nervous system activation was significantly more pronounced in the Wistar rats than the ETB rats. The SNS index of the Wistar rats rose during stress and remained elevated during recovery, but the difference was not statistically significant.*

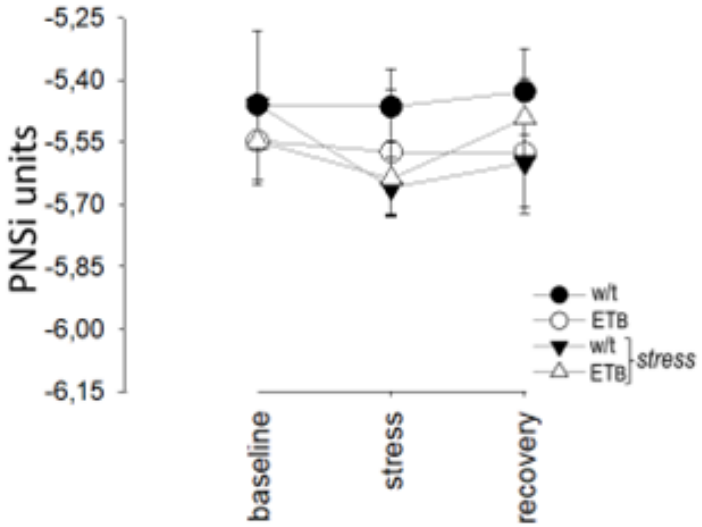
Table 8: *Sympathetic nervous system index recordings. The first line shows the mean value and standard deviation for each group ± SD. The rest of the table shows the p-value after the comparison between 2 groups (column - row). (SD=standard deviation). As can be seen from the table, there is a statistically significant difference during baseline, as well as during and post-stress, between the two genotypes with the Wistar rats having an over-activation of the SNS in comparison to the ETB rats. Significant p values are displayed in red.*

SNS INDEX

	Wistar	*ETB*	*Wistar stress*	*ETB stress*
	Baseline			
Mean of SNS index units ±SD	482.83 ± 213.10	327.57 ± 101.31	419.73 ± 217.89	324.26 ± 41.20
Wistar		0.1643	0.9900	0.1427
ETB	0.1643		0.8590	1.0000
Wistar stress	0.9900	0.8590		0.8288
ETB stress	0.1427	1.0000	0.8288	
	Stress period			
Mean of SNS index units ±SD	492.80 ± 129.22	311.92 ± 80.13	610.64 ± 111.18	339.17 ± 56.35
Wistar		0.0491	0.5630	0.1760
ETB	0.0491		0.0001	1.0000
Wistar stress	0.5630	0.0001		0.0002
ETB stress	0.1760	1.0000	0.0002	
	Recovery period			
Mean of SNS index units ±SD	442.53 ± 102.37	335.63 ± 96.71	544.04 ± 97.21	319.89 ± 37.33
Wistar		0.7028	0.7660	0.5003
ETB	0.7028		0.0103	1.0000
Wistar stress	0.7660	0.0103		0.0040
ETB stress	0.5003	1.0000	0.0040	

9.1.7. Vagal activity

PNS index was comparable between the two groups of Wistar rats at baseline, as it also was for the two ETB groups. When comparing the two genotypes at baseline no difference was observed. The PNSi during stress and recovery (expressed in PNSi units) is shown in Graph 6; PNSi remained stable in the absence of stress, but a significant variance was present in the stress groups; of note, in the Wistar rats vagal withdrawal was observed, to -5.659 ± 0.071 during stress and to -5.599 ± 0.121 during recovery, from -5.350 ± 0.169 in baseline ($p = 0.0001$). Moreover, in the ETB rats, during stress the PNS index was significantly lowered to -5.638 ± 0.088, from -5.512 ± 0.084 in baseline ($p = 0.0019$), but it seems to be restored during recovery. Between-group comparisons for the Wistar and the ETB rats revealed no significant difference in the rest of the study periods (Table 7). From Graph 6, it appears that there is a tendency of the PNSi to return to normal values post-stress.



Graph 7: *Parasympathetic nervous system response. Sympathetic nervous system activation was significantly lower in the ETB rats than the Wistar rats during normal conditions. The PNS index of the Wistar rats significantly decreased during stress and remained low during recovery. The PNSi of the ETB rats significantly decreased during stress, but returned to normal values during recovery.*

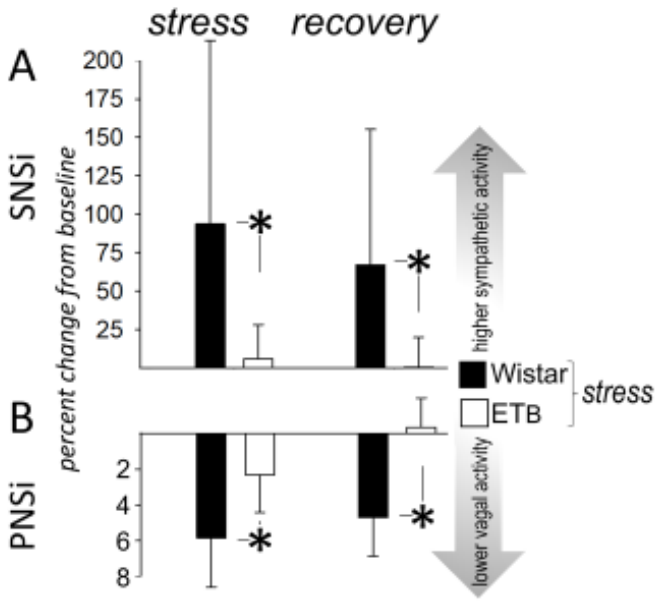
Table 9: *Parasympathetic nervous system index recordings. The first line shows the mean value and standard deviation for each group ± SD. The rest of the table shows the p-value after the comparison between 2 groups (column - row). (SD=standard deviation). As can be seen from the table, there is a statistically significant difference of the PNSi in wild-type rats during stress, in comparison to the absence of stress. Between groups comparison revealed no difference. Significant p values are displayed in red.*

PNS INDEX

	Wistar	*ETB*	*Wistar stress*	*ETB stress*
	Baseline			
Mean of PNS index units ±SD	-5.568 ± 0.115	-5.581 ± 0.118	-5.350 ± 0.169	-5.512 ± 0.084
Wistar		1.0000	0.0046	0.9944
ETB	1.0000		0.9999	0.9688
Wistar stress	0.0046	0.9999		0.0948
ETB stress	0.9944	0.9688	0.0948	
	Stress period			
Mean of PNS index units ±SD	-5.464 ± 0.092	-5.572 ± 0.151	-5.659 ± 0.071	-5.638 ± 0.088
Wistar		0.6174	0.0172	0.0533
ETB	0.6174		0.8652	0.9787
Wistar stress	0.0172	0.8652		1.0000
ETB stress	0.0533	0.9787	1.0000	
	Recovery period			
Mean of PNS index units ±SD	-5.427 ± 0.105	-5.576 ± 0.130	-5.599 ± 0.121	-5.492 ± 0.095
Wistar		0.1710	0.0595	0.9810
ETB	0.1710		1.0000	0.8869
Wistar stress	0.0595	1.0000		0.6331
ETB stress	0.9810	0.8869	0.6331	

9.1.8. Sympatho-vagal balance during stress

The activation of the SNS during stress is significantly elevated in the Wistar group when compared to the ETBs, while the activation of the PNS lowers significantly in the w/t rats (Graph 8). In recovery, the SNS of the Wistar rats is, again, significantly elevated in comparison to the ETB rats. The PNS percent change during recovery is statistically different between the two groups. As Graph 7 presents, in recovery, the PNS activation of the Wistar rats is lowered, but the PNS activation of the ETB rats shows a small increase in comparison to the baseline values of the same group. Tables 8 and 9 present the % changes of SNS and PNS, respectively, of each group and the p-values from the comparison between them.



Graph 8: *Sympatho-vagal balance. The graph shows percent change of SNSi and PNSi from baseline values during stress and recovery, for the two rat genotypes. The Wistar rats have significantly higher sympathetic activity and significantly lower vagal activity than the ETB rats both during stress and recovery (asterisks).*

Table 10: *Sympathetic nervous system activation percent change from baseline. The first two columns show the % change of the SNS activation during stress and recovery. The third column shows the p-value after the comparison between 2 groups (Wistar-ETB). Significant p values are displayed in red.*

SNS CHANGE FROM BASELINE (%)			
	WISTAR	**ETB**	**p-value**
Stress	93.9 ↑	59.9 ↑	0.0189
Recovery	67.0 ↑	0.6 ↑	0.0184

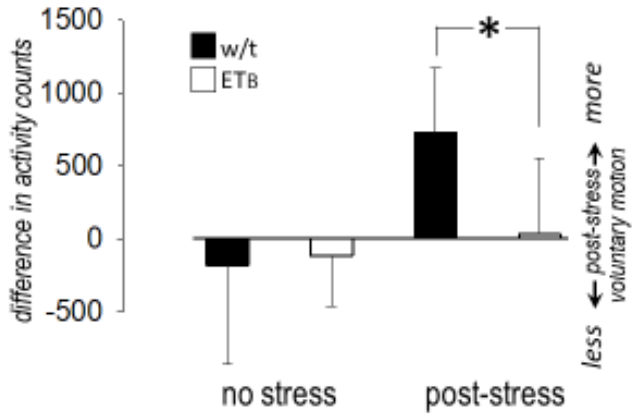
Table 11: *Parasympathetic nervous system activation percent change from baseline. The first two columns show the % change of the PNS activation during stress and recovery. The third column shows the p-value after the comparison between 2 groups (Wistar-ETB). Significant p values are displayed in red.*

PNS CHANGE FROM BASELINE (%)			
	WISTAR	**ETB**	**p-value**
Stress	58.4 ↓	23.0 ↓	0.0016
Recovery	46.9 ↓	0.4 ↑	0.0002

9.1.9. Voluntary activity

The two groups showed no difference between them in the absence of acute emotional stress, as can be seen in Graph 9, in terms of spontaneous motor activity. In contrast, voluntary motion post-stress was significantly more pronounced in the Wistar rats, which is a rough indicator of emotional stress or heart failure. Emotional stress had no effect on voluntary motion during recovery in ETB-deficient rats. In more detail, the number of motor counts (expressed as the total

movements of the rat in 1 hour) for each group, as well as the p-values from the comparison between the groups are recorded in Table 10.



Graph 9: *Recording of the difference in spontaneous motor activity of the rats. Voluntary motion post-stress was significantly higher in the Wistar in comparison to the ETB rats (asterisk).*

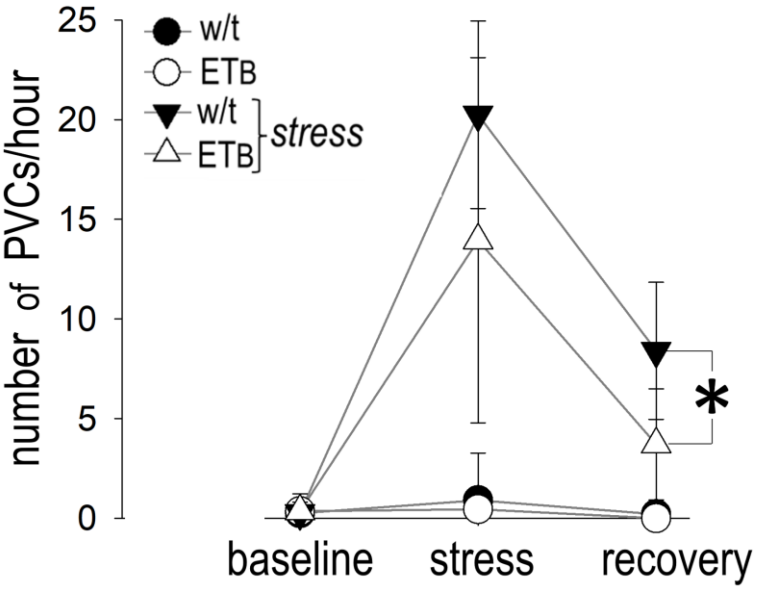
Table 12: *Voluntary motion of the rats. The table shows the mean and standard deviation for each group.*

	VOLUNTARY MOTION			
	WISTAR	*ETB*	*WISTAR STRESS*	*ETB STRESS*
MEAN OF MOVEMENTS/HOUR ± SD	444 ± 572	477 ± 428	778 ± 242	496 ± 219

9.1.10. Arrhythmias

9.1.10.1. Tachyarrhythmias

Apart from some simple spontaneous ventricular contractions, no episodes of VTs/VFs were recorded in the animals of first part of the study. All four groups developed no PVCs during the baseline recordings. As shown in Graph 10 both the Wistar and the ETB rats had a significantly elevated number of PVCs during stress and recovery, in comparison to the baseline. During stress, there was a statistical trend (p=0.064), but not significance, towards more frequent PVCs in wild-type rats, when compared to ETB-deficient rats. However, this difference became significant during the recovery period, with the Wistar rats having a larger number of premature ventricular contractions than the ETB rats. The number of PVCs per hour as well as the p-values after the comparison of the groups is shown in Table 11. No Couplets and Triplets were recorded during the hours of the observation in all four groups.



Graph 10: *Recording of spontaneous ventricular contractions (PVCs) per hour of the rats. PVCs number post-stress was significantly higher in the Wistar in comparison to the ETB rats (asterisk).*

Table 13: *Spontaneous ventricular contractions. The first line shows the mean number and standard deviation for each group ± SD. The rest of the table shows the p-value after the comparison between 2 groups (column - row). (SD=standard deviation). As can be seen from the table, there is a statistically significant difference post-stress, between the two genotypes and in comparison to the baseline values. Significant p values are displayed in red.*

SPONTANEOUS VENTRICULAR CONTRACTIONS (PVCs)

	Wistar	*ETB*	*Wistar stress*	*ETB stress*
	Baseline			
Mean number of PVCs/hour ±SD	0 ± 0	1 ± 0	0 ± 1	1 ± 1
Wistar		1.0000	1.0000	1.0000
ETB	1.0000		1.0000	1.0000
Wistar stress	1.0000	1.0000		1.0000
ETB stress	1.0000	1.0000	1.0000	
	Stress period			
Mean number of PVCs/hour ±SD	1 ± 2	1 ± 1	20 ± 5	14 ± 9
Wistar		1.0000	0.0001	0.0001
ETB	1.0000		0.0001	0.0001
Wistar stress	0.0001	0.0001		0.0642
ETB stress	0.0001	0.0001	0.0642	
	Recovery period			
Mean number of PVCs/hour ±SD	0 ± 1	0 ± 0	17 ± 7	7 ± 6
Wistar		1.0000	0.0001	0.0094
ETB	1.0000		0.0001	0.0045
Wistar stress	0.0001	0.0001		0.0001
ETB stress	0.0094	0.0045	0.0001	

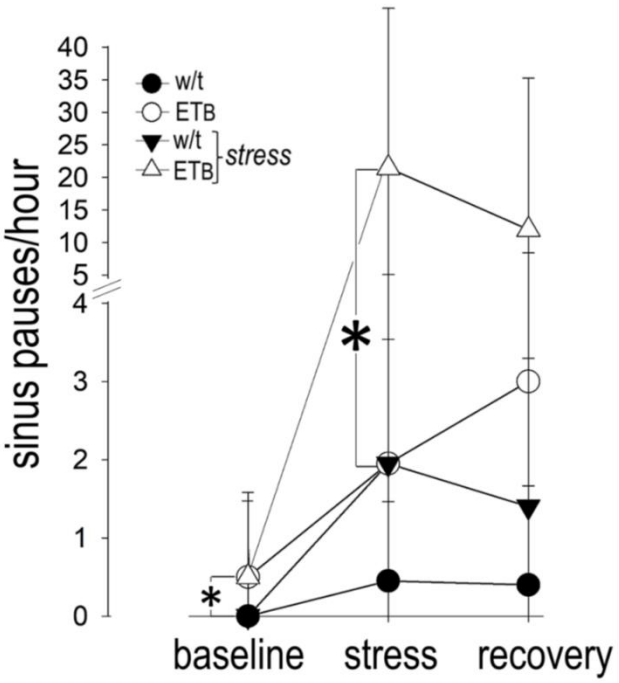
9.1.10.2. Bradyarrhythmias

AV blocks

A statistically insignificant number of AV blocks was recorded during the stress period in both Wistar and ETB rats (3 ± 6 and 2 ± 1, respectively), while in the rest of the recording periods no more AV blocks were found.

Sinus pauses

As shown on Graph 11, both rat genotypes do not develop sinus pauses during normal circumstances. In the setting of acute stress, though, ETB rats appear to have a significant rise of the number of sinus pauses developed (22 ± 25 from 1 ± 1, p = 0.0017). When compared to the Wistar rats during stress, it is shown that this rise of the sinus pauses number is also statistically significant (p = 0.0017). Sinus pauses in the ETB group continue to happen, in a reduced number, post-stress, but with no statistical significance. Table 12 lists the mean number of sinus pauses for each experimental group in each study period, as well as all the p-values. An example of sinus pause is shown in Figure 25.



Graph 11: *Recording of sinus pauses per hour, of the rats. Sinus pauses number significantly rose during the stress period. Moreover, during stress, the number of sinus pauses was significantly higher in the ETB in comparison to the Wistar rats (asterisk).*

Table 14: *Sinus pauses. The first line of each period shows the mean number and standard deviation for each group ± SD. The rest of the table shows the p-value after the comparison between 2 groups (column - row). (SD=standard deviation). As can be seen from the table, there is a statistically significant difference during stress, between the two genotypes and in comparison to the baseline values for the ETB rats, which appear to develop a significant number of sinus pauses. Significant p values are displayed in red.*

SINUS PAUSES

	Wistar	*ETB*	*Wistar stress*	*ETB stress*
	Baseline			
Mean number of sinus pauses/hour ±SD	0 ± 0	1 ± 1	0 ± 0	1 ± 1
Wistar		1.0000	1.0000	1.0000
ETB	1.0000		1.0000	1.0000
Wistar stress	1.0000	1.0000		1.0000
ETB stress	1.0000	1.0000	1.0000	
	Stress period			
Mean number of sinus pauses/hour ±SD	1 ± 1	2 ± 3	2 ± 2	22 ± 25
Wistar		1.0000	1.0000	0.0006
ETB	1.0000		1.0000	0.0017
Wistar stress	1.0000	1.0000		0.0017
ETB stress	0.0006	0.0017	0.0017	
	Recovery period			
Mean number of sinus pauses/hour ±SD	0 ± 1	3 ± 5	1 ± 2	12 ± 23
Wistar		1.0000	1.0000	0.2916
ETB	1.0000		1.0000	0.6795
Wistar stress	1.0000	1.0000		0.4298
ETB stress	0.2916	0.6795	0.4298	

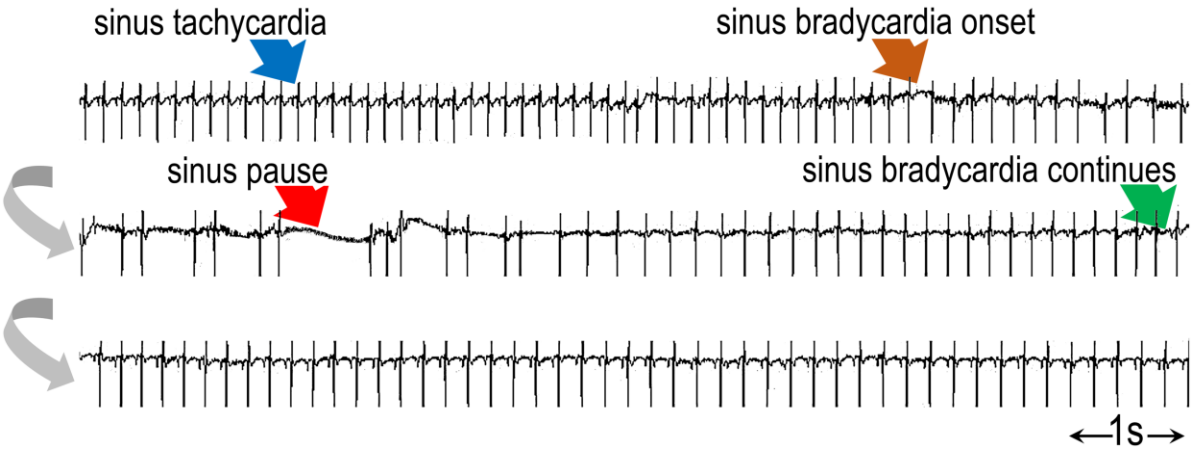


Figure 25. *Example of sinus pause in an ETB-deficient rat. Note the sinus tachycardia (~400bpm, blue arrow) preceding the onset of sinus bradycardia (brown arrow), leading to a sinus pause (red arrow). Sinus bradycardia with discernible P waves resumes later (green arrow).*

9.2. SECOND PART RESULTS

This part of the experimental study included 23 male Wistar (w/t) rats and 24 male ETB deficient rats, aged 18-20 weeks and weighing 376±61 g. From the 47 rats of this part, 3 Wistar and 4 ETB deficient died during or after the procedure and were excluded from the study. The rest survived until their sacrifice and took part in the evaluation of the heart rate variability and arrhythmias and had a permanent ligation of the left coronary artery surgery. Each experimental group of the evaluation of the heart rate variability and arrhythmias included 10 rats. The average weight of the Wistar rats was 438±35 g, while the average weight of the ETB deficient rats was 277±48 g. For the two different genotypes, after randomization, a stress group and a control group (no stress) where formatted.

9.2.1. Mortality

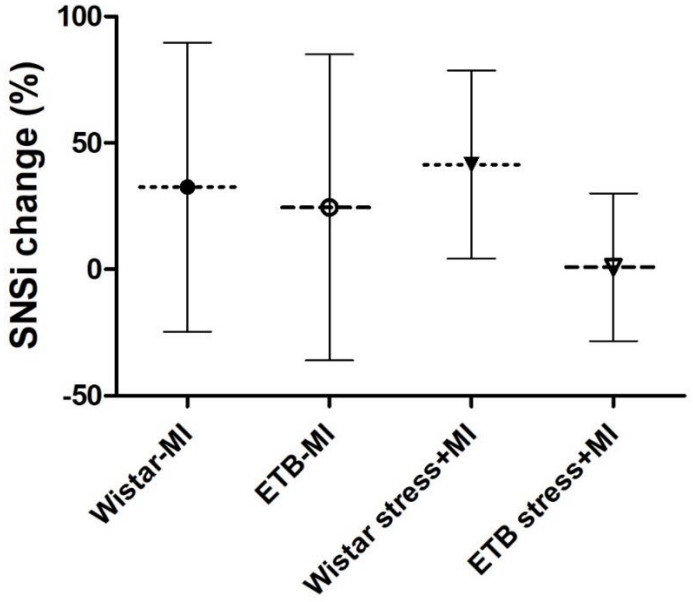
As mentioned before, 3 Wistar rats, of which 2 had ligation and 1 experienced acute emotional stress before the ligation died during or after the surgery. In addition, 4 ETB deficient rats, of which 2 had ligation and 2 experienced acute emotional stress before the ligation died during or after the surgery. There was no difference in the mortality between the four groups and the mortality observed (9.1% for the Wistar no-stress group and 16.6% for the other three groups) is among the expected mortality after myocardial infarction.

9.2.2. Infarct size

Infarct size was calculated for 20 (10 Wistar and 10 ETB deficient) survivors, which were selected after randomization, and was comparable between the two groups (37.9% ± 1.5% and 39.1% ± 1.4% respectively, p=0.5712).

9.2.3. Sympathetic activity

SNS index was elevated after the onset of myocardial infarction in both the Wistar groups (Wistar-MI and Wistar stress + MI) and the ETB deficient rats without previous stress, in comparison to the baseline values. This rise did not appear in the ETB deficient group when stress preceded the ligation of the left coronary artery (Graph 12). Between-group comparisons for the Wistar and the ETB rats with myocardial infarction, in the presence or not of acute emotional stress, revealed no significant differences in the SNSi change from baseline values, even though SNSi of the ETB stress + MI group only rose 0.9% while the rise in the other groups was much higher (Table 15).



Graph 12: *The graph shows the percent change of SNSi from baseline values during the eight-hour recording period post- myocardial infarction (MI), for the four rat groups. The SNS index of the ETB rats with stress and MI did not change post-MI, but the difference was not statistically significant in comparison to the other groups. Vertical bars denote the standard deviation.*

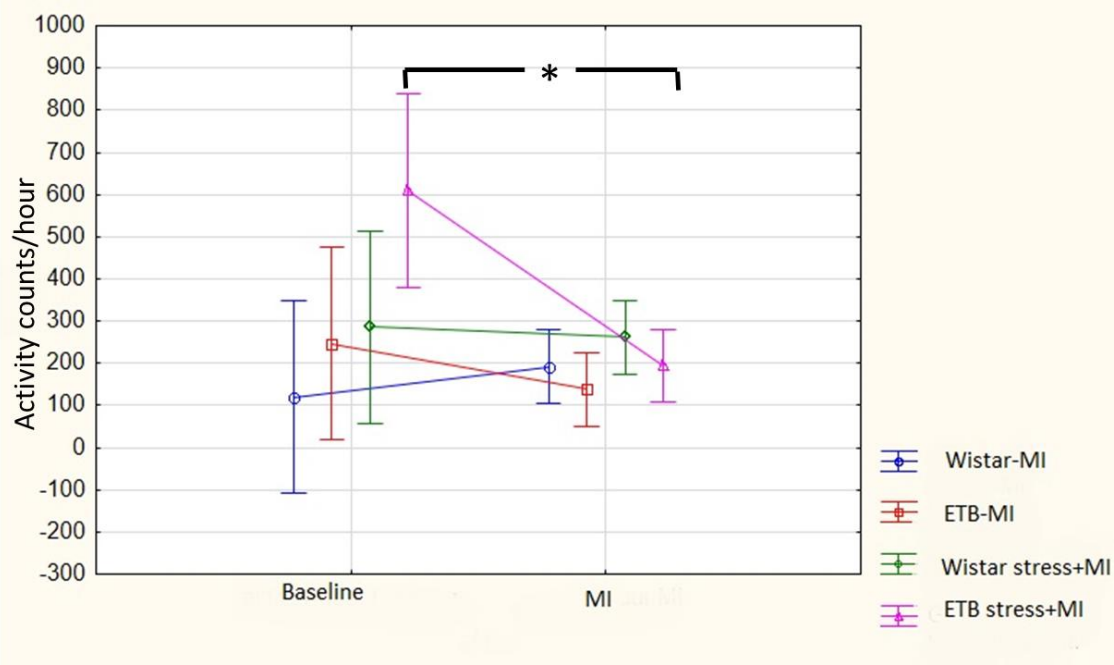
Table 15: *Sympathetic nervous system activation percent change from baseline. The SNSi activation is calculated for eight-hour-post-MI recordings. The first line of the table shows the SNS change from baseline (%). The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, there is no statistically significant difference between the four groups. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.*

SNS CHANGE FROM BASELINE (%)

	Wistar MI	*ETB MI*	*Wistar stress + MI*	*ETB stress + MI*
	Post-MI			
SNS CHANGE FROM BASELINE ± SD (%)	32.5% ± 57.1%	24.6% ± 60.5%	41.4% ± 37.2%	0.9% ± 29.2%
Wistar MI		0.9821	0.9758	0.4615
ETB MI	0.9821		0.8601	0.6893
Wistar stress + MI	0.9758	0.8601		0.2500
ETB stress + MI	0.4615	0.6893	0.2500	

9.2.4. Voluntary activity

The two groups showed no difference between them in the absence of acute emotional stress, as can be seen in Graph 9, in terms of spontaneous motor activity. In contrast, voluntary motion post-stress was significantly more pronounced in the Wistar rats, which is a rough indicator of emotional stress or heart failure. Emotional stress had no effect on voluntary motion during recovery in ETB-deficient rats. In more detail, the number of motor counts (expressed as the total movements of the rat in 1 hour) for each group, as well as the p-values from the comparison between the groups are recorded in Table 10.



Graph 13: *Recording of the difference in spontaneous motor activity of the rats per hour. Voluntary motion post-MI was significantly lower in the ETB deficient rats with the presence of acute emotional stress, in comparison to their baseline activity (asterisk). Vertical bars denote 0.95 confidence intervals.*

Table 16: *Voluntary motion of the rats in baseline and post-MI. The table shows the mean and standard deviation for each group. SD= Standard Deviation.*

	Baseline	*MI*
	ACTIVITY COUNTS/HOUR ± SD	
Wistar MI	120 ± 151	193 ± 178
ETB MI	247 ± 239	139 ±111
Wistar stress + MI	286 ± 437	262 ± 107
ETB stress + MI	609 ± 489	194 ± 135

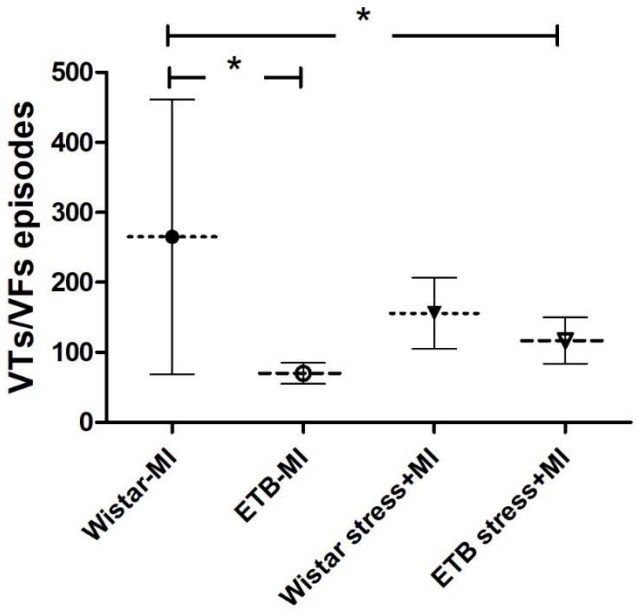
9.2.5. Arrhythmias

9.2.5.1. Tachyarrhythmias

The number of tachyarrhythmias, of the second part of the study, is presented as sum of the number of episodes or sum of the duration of those episodes recorded during the eight-hour observational period post-MI. Apart from some simple spontaneous ventricular contractions, no episodes of VTs/VFs were recorded during baseline. All four groups developed tachyarrhythmic episodes in the form of VTs/VFs, premature ventricular contractions, couplets and triplets during the eight-hour observational period post-MI recordings.

Ventricular tachycardias / Ventricular Fibrillations (VTs/VFs)

As shown in Graph 14 all the four groups had VTs/VFs during their post-MI recovery. Both the ETB deficient groups had a significantly lower number of PVCs post-MI, in comparison to the wild-type rats with MI but without acute emotional stress. In detail, the Wistar-MI group had 265 episodes of VTs/VFs with a total duration of 1006 seconds, while the ETB-MI and the ETB stress + MI groups had 70 episodes with 199.8 seconds duration and 117 episodes with 272.7 seconds duration respectively. The same difference is also observed in the total duration of those episodes (Graph 15). There was no difference in the number or the duration of VTs/VFs in the presence of stress in comparison to its absence in both the wild-type and the ETB deficient rats. The number of the total VTs/VFs as well as the p-values after the comparison of the groups is shown in Table 17, while the duration of those episodes is recorded in Table 18.

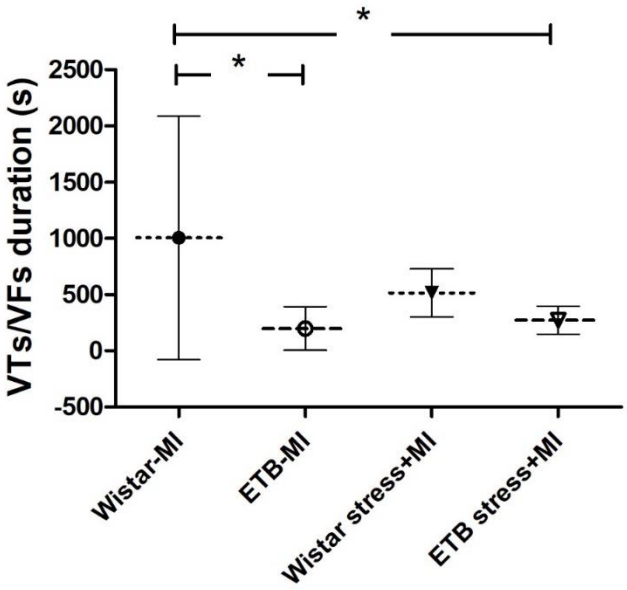


Graph 14: *Recording of the number of ventricular tachycardias/ventricular fibrillations (VTs/VFs) post-MI. VFs/VTs number post-MI was significantly higher in the Wistar no-stress rats in comparison to the ETB deficient rats (asterisks). Vertical bars denote the standard deviation.*

Table 17: *Number of ventricular tachycardias/ventricular fibrillations (VTs/VFs) post-MI. The number of VTs/VFs is calculated as the sum of episodes of eight-hour-post-MI recordings. The first line of the table shows the number of VTs/VFs episodes recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, the wild-type rats with only myocardial infarction had significantly more VTs/VFs episodes than the two ETB deficient groups. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.*

VTs/VFs EPISODES

	Wistar MI	*ETB MI*	*Wistar stress + MI*	*ETB stress + MI*
	Post-MI			
Number of VTs/VFs episodes ± SD	265 ± 196	70 ± 15	156 ± 51	117 ± 33
Wistar MI		0.0011	0.1025	0.0142
ETB MI	0.0011		0.2593	0.7388
Wistar stress + MI	0.1025	0.2593		0.8311
ETB stress + MI	0.0142	0.7388	0.8311	



Graph 15: *Recording of the total duration of ventricular tachycardias/ventricular fibrillations (VTs/VFs) post-MI in seconds. VFs/VTs total duration post-MI was significantly higher in the Wistar no-stress rats in comparison to the ETB deficient rats (asterisks). Vertical bars denote standard deviation*

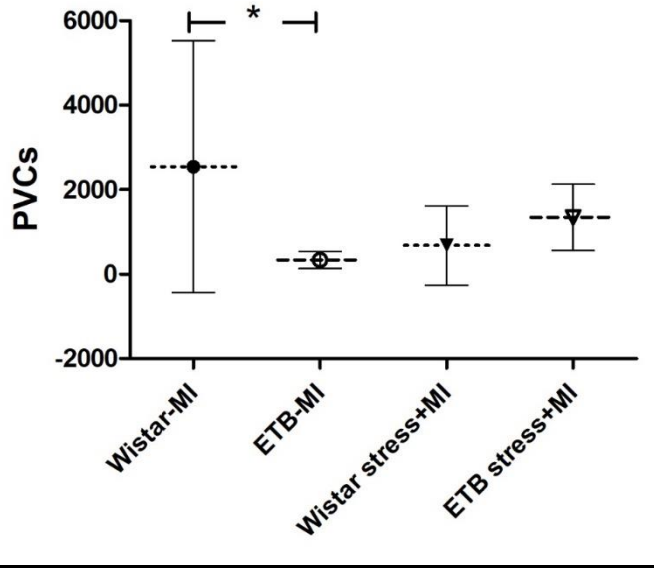
Table 18: *Total duration of ventricular tachycardias/ventricular fibrillations (VTs/VFs) post-MI in seconds. The duration of VTs/VFs is calculated as the sum of the duration of each episode of the eight-hour-post-MI recordings. The first line of the table shows the total duration of VTs/VFs episodes recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, the wild-type rats with only myocardial infarction had significantly higher total duration of VTs/VFs episodes than the two ETB deficient groups. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.*

TOTAL DURATION OF VTs/VFs

	Wistar MI	*ETB MI*	*Wistar stress + MI*	*ETB stress + MI*
	Post-MI			
Total duration of VTs/VFs episodes ± SD (sec.)	1006.0 ± 1081.5	199.8 ± 193.3	516.7 ± 212.5	272.7 ± 124.6
Wistar MI		0.0146	0.2284	0.0299
ETB MI	0.0146		0.5946	0.9915
Wistar stress + MI	0.2284	0.5946		0.7677
ETB stress + MI	0.0299	0.9915	0.7677	

Premature ventricular contractions (PVCs)

As shown in Graph 16 both the Wistar and the ETB rats had PVCs during the eight-hour post-MI recovery. During this period, the presence of PVCs was significantly more frequent in the wild-type rats (2540 episodes), when compared to ETB-deficient rats (335 episodes), in the absence of stress with a p value = 0.0211. However, this difference did not appear between the first group and the ETB deficient rats with stress, nor between the same strain groups. The number of the total PVCs as well as the p-values after the comparison of the groups is shown in Table 19.



Graph 16: *Recording of spontaneous ventricular contractions (PVCs) post-MI. PVCs number post-MI was significantly higher in the Wistar-MI rats in comparison to the ETB deficient rats (ETB-MI), in the absence of stress (asterisk). Vertical bars denote the standard deviation.*

Table 19: *Total number of premature ventricular contractions (PVCs) post-MI. The number of PVCs is calculated as the sum of the number of each PVC episode of the eight-hour-post-MI recordings. The first line of the table shows the total number of PVCs recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, the wild-type rats with myocardial infarction had significantly higher total number of PVCs than*

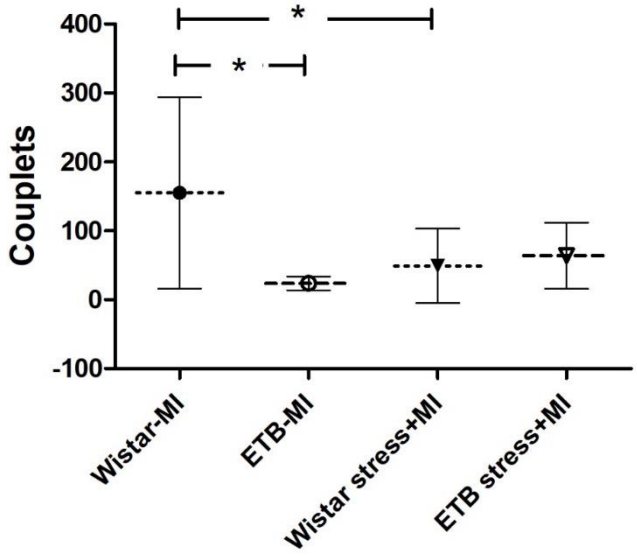
the ETB deficient rats with myocardial infarction, in the absence of acute emotional stress. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.

SPONTANEOUS VENTRICULAR CONTRACTIONS (PVCs)

	Wistar MI	*ETB MI*	*Wistar stress + MI*	*ETB stress + MI*
	Post-MI			
Number of PVCs ± SD	2540 ± 2980	335 ± 199	680 ± 939	1346 ± 785
Wistar MI		0.0211	0.0650	0.3618
ETB MI	0.0211		0.9637	0.5078
Wistar stress + MI	0.0650	0.9637		0.7931
ETB stress + MI	0.3618	0.5078	0.7931	

Couplets

As shown in Graph 17 both the Wistar and the ETB rats had couplets (two consecutive PVCs) during the eight-hour post-MI recovery. During this period, the presence of couplets was significantly more frequent in the wild-type rats (155 episodes), when compared to ETB-deficient rats (24 episodes), in the absence of stress with a p value = 0.0073. Moreover, in the presence of stress before the myocardial infarction in the wild-type rats, the number of couplets significantly lowers. This difference did not appear between the ETB deficient groups. In addition, between strains comparison revealed no difference in the presence of stress between the two genotypes. The number of the total couplets as well as the p-values after the comparison of the groups is shown in Table 20.



Graph 17: *Recording of couplets post-MI. Couplets number post-MI was significantly higher in the Wistar in comparison to the ETB rats, in the absence of stress (asterisk). Wistar rats in the presence of stress had significantly less couplets than they had in its absence (asterisk). Vertical bars denote the standard deviation.*

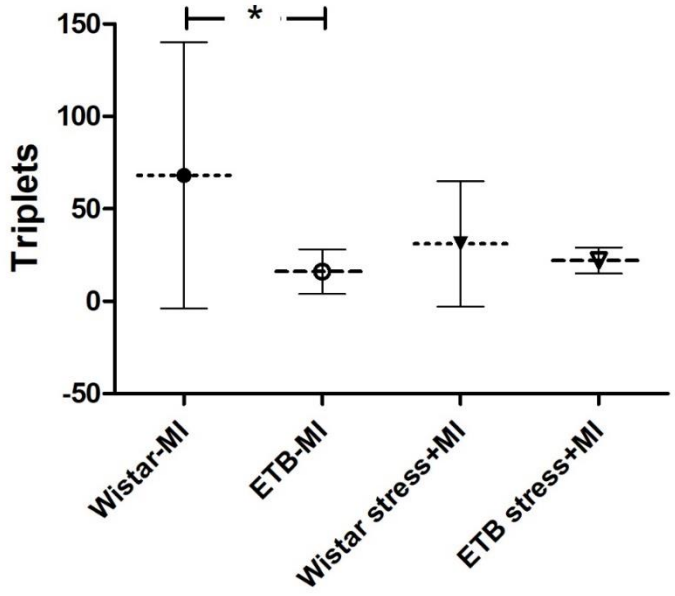
Table 20: *Total number of couplets, post-MI. The number of couplets is calculated as the sum of the number of each couplet episode of the eight-hour-post-MI recordings. The first line of the table shows the total number of couplets recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, the wild-type rats with myocardial infarction had significantly higher total number of couplets than the ETB deficient rats with myocardial infarction, in the absence of acute emotional stress. Moreover, post-MI, the Wistar rats had significantly less couplets in the presence of stress in comparison to its absence. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation*

COUPLETS

	Wistar MI	*ETB MI*	*Wistar stress+MI*	*ETB stress + MI*
	Post-MI			
Number of Couplets ± SD	155 ± 139	24 ± 10	49 ± 54	64 ± 48
Wistar MI		0.0073	0.0444	0.1095
ETB MI	0.0073		0.8872	0.6700
Wistar stress + MI	0.0444	0.8872		0.9760
ETB stress + MI	0.1095	0.6700	0.9760	

Triplets

As shown in Graph 18 both the Wistar and the ETB rats had triplets (three consecutive PVCs) during the eight-hour post-MI recovery. During this period, the presence of triplets was significantly more frequent in the wild-type rats (68 episodes), when compared to ETB-deficient rats (16 episodes), in the absence of stress with a p value = 0.0372. However, this difference did not appear between the first group and the ETB deficient rats with stress, nor between the same strain groups. The number of the total PVCs as well as the p-values after the comparison of the groups is shown in Table 21.



Graph 18: *Recording of triplets, post-MI. Triplets number post-MI was significantly higher in the Wistar-MI rats in comparison to the ETB deficient rats (ETB-MI), in the absence of stress (asterisk). Vertical bars denote the standard deviation.*

Table 21: *Total number of triplets, post-MI. The number of triplets is calculated as the sum of the number of each triplet episode of the eight-hour-post-MI recordings. The first line of the table shows the total number of triplets recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, the wild-type rats with myocardial infarction had significantly higher total number of triplets than the ETB deficient*

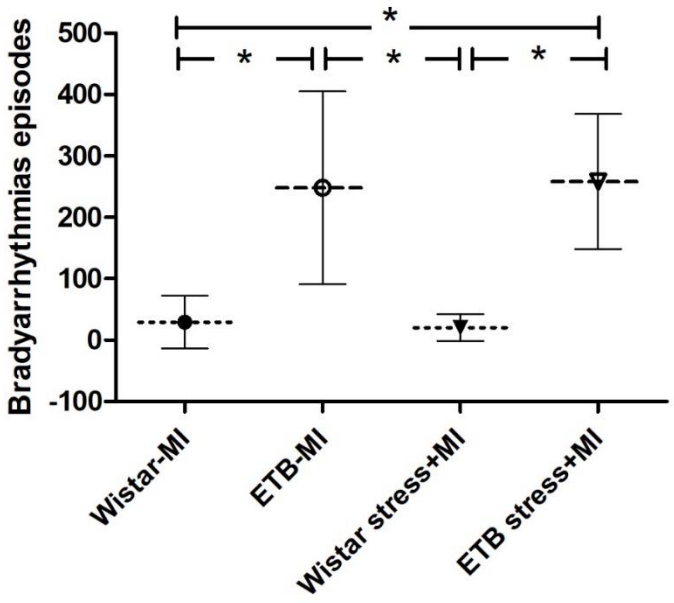
rats with myocardial infarction, in the absence of acute emotional stress. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.

TRIPLETS

	Wistar MI	*ETB MI*	*Wistar stress+MI*	*ETB stress + MI*
	Post-MI			
Number of Triplets + SD	68 ± 72	16 ± 12	31 ± 34	22 ± 7
Wistar MI		0.0372	0.2079	0.0855
ETB MI	0.0372		0.8414	0.9909
Wistar stress + MI	0.2079	0.8414		0.9565
ETB stress + MI	0.0855	0.9909	0.9565	

9.2.5.2. Bradyarrhythmias

As shown in Graph 19 all the four groups had bradyarrhythmic episodes (sinus pauses and AV blocks) during their post-MI recovery. In the ETB deficient rats sinus pauses were more frequent than atrio-ventricular blocks. Both the ETB deficient groups had a significantly higher number and duration of bradyarrhythmias post-MI, in comparison to the wild-type rats both in the presence or the absence of acute emotional stress. In detail, the Wistar-MI group had 29 episodes of VTs/VFs with a total duration of 249.6 seconds, while the ETB-MI and the ETB stress + MI groups had 249 episodes with 946.5 seconds duration and 258 episodes with 822.9 seconds duration respectively. The same difference is also observed in the total duration of those episodes (Graph 20). There was no difference in the number or the duration of bradyarrhythmias in the presence of stress in comparison to its absence in both the wild-type and the ETB deficient rats. The number of the total bradyarrhythmic episodes as well as the p-values after the comparison of the groups is shown in Table 22, while the duration of those episodes is recorded in Table 23.

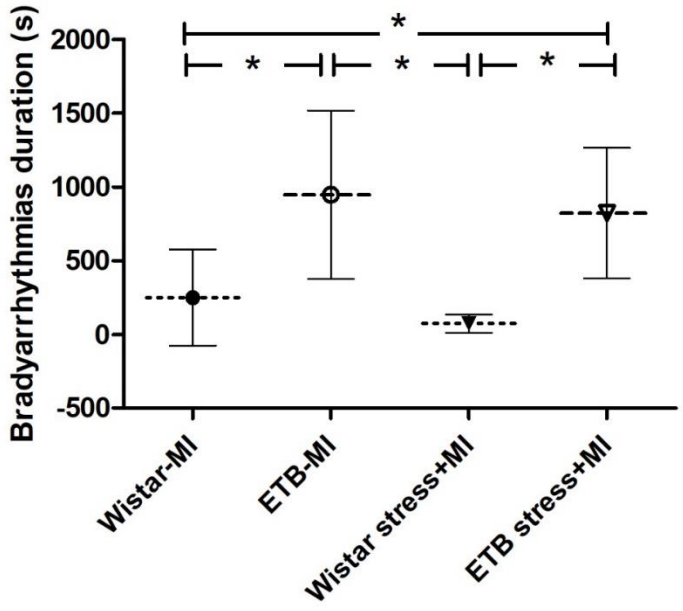


Graph 19: *Recording of the number of bradyarrhythmias, post-MI. Bradyarrhythmic episodes number post-MI was significantly lower in the Wistar rats in comparison to the ETB deficient rats both in the absence or the presence of stress (asterisks). Vertical bars denote the standard deviation.*

Table 22: *Number of bradyarrhythmic episodes, post-MI. The number of bradyarrhythmias is calculated as the sum of the total episodes (sinus pauses and AV blocks) of eight-hour-post-MI recordings. The first line of the table shows the number of bradyarrhythmic episodes recorded. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, both the ETB deficient groups have significantly elevated incidents of bradyarrhythmias, in comparison to the wild-type rats, post-MI. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.*

BRADYARRHYTHMIC EPISODES

	Wistar MI	*ETB MI*	*Wistar stress+MI*	*ETB stress + MI*
	Post-MI			
Number of bradyarrhythmic episodes ± SD	29 ± 43	248 ± 157	20 ± 22	258 ± 110
Wistar MI		0.0003	0.9969	0.0002
ETB MI	0.0003		0.0002	0.9957
Wistar stress + MI	0.9969	0.0002		0.0002
ETB stress + MI	0.0002	0.9957	0.0002	



Graph 20: *Recording of the duration of bradyarrhythmias (in seconds), post-MI. Total bradyarrhythmic episodes duration post-MI was significantly lower in the Wistar rats in comparison to the ETB deficient rats both in the absence or the presence of stress (asterisks). Vertical bars denote the standard deviation.*

Table 23: *Total duration of bradyarrhythmic episodes, post-MI. The duration of bradyarrhythmias is calculated as the sum of each episodes' duration (sinus pauses and AV blocks) of eight-hour-post-MI recordings. The first line of the table shows the duration of bradyarrhythmic episodes recorded, in seconds. The rest of the table shows the p-value after the comparison between 2 groups (column - row). As can be seen from the table, both the ETB deficient groups have significantly higher total duration of bradyarrhythmias, in comparison to the wild-type rats, post-MI. Significant p values are displayed in red. MI= myocardial infarction, SD= Standard Deviation.*

BRADYARRHYTHMIC EPISODES DURATION

	Wistar MI	*ETB MI*	*Wistar stress+MI*	*ETB stress + MI*
	Post-MI			
Total duration of bradyarrhythmic episodes ± SD (sec.)	249.6 ± 326.5	946.5 ± 570.1	73.2 ± 61.3	822.9 ± 442.0
Wistar MI		0.0022	0.7542	0.0136
ETB MI	0.0022		0.0003	0.8979
Wistar stress + MI	0.7542	0.0003		0.0010
ETB stress + MI	0.0136	0.8979	0.0010	

10. DISCUSSION

Central sympathetic activation occurs after acute coronary occlusion and may contribute to arrhythmogenesis **(44)**. This response may be exaggerated in the setting of acute emotional stress (AES) prior to **(171)**, or shortly after, the onset of myocardial ischemia **(167)**. Yet, the evoked electrophysiological alterations and the factors modulating central sympathetic inputs remain poorly understood. Recently accumulated evidence implicates ET-1 as an important modulator of acute and chronic sympathetic activation. In addition to its direct arrhythmogenic actions, endothelin may mediate autonomic effects on cardiac electrophysiology, and, may, thereby, play a role in the pathogenesis of rhythm disturbances. However, the role of endothelin and its receptors in these processes remains obscure. This dissertation was designed to investigate the pathophysiologic role of ET-1 in acute emotional stress and to shed light into the pathophysiology of ischemia-induced arrhythmogenesis, thus the study was separated in two parts, respectively.

10.1. FIRST PART MAIN FINDINGS

The experiments of the first part of the study demonstrate sympathetic activation and vagal withdrawal in response to acute emotional stress in wild-type rats. As a result, rises in HR and BP at the range of 20-30% were recorded, which are comparable to those reported in a similar protocol **(253)**. By contrast, autonomic responses were blunted in ETB-deficient rats, especially regarding the sympathetic arm. The markedly different patterns of autonomic activity did not yield differences in HR and BP, but voluntary activity during recovery was higher in wild-type rats, reflecting prolonged sympathetic activation in this strain. More importantly, the blunted sympathetic response in ETB-deficient rats was reflected in frequent bradyarrhythmias during acute emotional stress and recovery, contrasting the frequent PVCs observed in wild-type rats.

10.1.1. Autonomic responses in wild-type rats

Despite the widespread use of the rat-model of AES, detailed evaluation of sympathetic and vagal responses is scarce. Analyzing time-domain parameters of HRV, Sgoifo et al **(254)** described higher sympathetic activation with simultaneous lower vagal antagonism during a 15 min recording period in a model of social stress in rats, a model relevant to depressive and anxiety disorders. Together with our initial experience **(198)**, the HRV analysis of this study provides an important addition to the characterization of the present AES-protocol, which is considered more relevant to emotions of fear **(255-257)**. Moreover, the evolvement of HRV analysis with the combination of several variables derived from frequency domain and nonlinear analysis, as used here, provides a more accurate description of autonomic changes over short periods of time **(258)**.

Rapid activation of the sympathetic arm was observed at the onset of stress in wild-type rats, with a subsequent plateau at lower values until the end of AES and recovery. The initial rise in sympathetic activation was coupled with vagal withdrawal; although relatively less pronounced, vagal changes were sustained during the entire observational period and mostly accounted for the continuing anxiety during recovery in wild-type rats. Thus, in keeping with previous findings in rats **(259)** and humans **(260)**, the findings of this study underline the prominent role of vagal withdrawal after AES. Indeed, the role of vagal activity is thought to be critical in the shift to fight-or-flight actions in response to acute stressors of varying intensity **(261)**.

10.1.2. Observational period duration

The main aim of the present work was the investigation of the pathophysiologic role of ET-1 in AES. The role of ET-1 in this setting is likely two-fold, acting both at central **(262)** sites, as well as in the heart **(263)** and the vascular endothelium **(264)**. The rapid initial autonomic responses appear to be mediated by the brain central actions of ET-1, which acts at various sites in the brain **(253)**. ET-1 subsequently rises at the setting of AES, primarily secreted from

the vascular endothelium **(205)**, an action mediated by corticotropin releasing hormone, which has a central role in eliciting neuroendocrine, autonomic, and behavioral responses to stress **(265)**. Therefore, we opted for a long observation period (including 43 min AES and a two-hour recovery period), which permits comprehensive evaluation of the effects of ET-1.

10.1.3. Baseline autonomic characteristics of ETB-deficient rats

We utilized the rescued homozygous ETB receptor-deficient rat-strain, with absence of functioning ETB receptors in the cardiovascular system. This rat strain has been a valuable model in many studies investigating the role of chronically elevated high plasma ET-1 levels. The comparison of continuous recordings prior to the induction of stress between wild-type and ETB-deficient rats provides further characterization of this strain. Despite the similar HR and BP, ETB-deficient rats had lower sympathetic and higher vagal activity at baseline, reinforcing previous observations of enhancement of vagal reflexes by ET-1, acting either centrally **(262)** or at peripheral sites of the reflex arc **(264)**. Interestingly, occasional sinus pauses were recorded in ETB-deficient but not in wild-type rats, in keeping with the previously reported inhibitory actions of ET-1 on calcium current in the rabbit sinus node **(263)**.

10.1.4. Sympathetic responses to stress in ETB-deficient rats

The most important finding of the present study was the different responses to AES in ETB-deficient rats, thereby reiterating the role of ET-1 in this setting. This strain displayed the blunted sympathetic response to AES throughout the observation period. This result contrasts the markedly enhanced sympathetic activation in this rat-strain, observed during global **(266)** or regional **(126)** ischemia, or in the setting of heart failure due to pressure overload **(267)**. However, due to the much larger scale of sympathetic activation during these

pathophysiologic states, they cannot be compared with the experimental conditions in the present work, obtained under conditions of normal myocardium.

Our results are in line with those reported in an experimental setting similar to the present study, in which enhanced baseline ET-1 levels by means of prior high salt diet in wild-type and ETB-deficient rats abrogated the pressor response to air-jet stress **(240)**. Likewise, diverse pressor responses were reported after AES in wild-type and ETB-deficient rats in the background of chronic behavioral stress, which was induced by early life stress **(268)**. In the latter work, wild type rats had increased circulating levels of ET-1 after early life stress and exhibited enhanced blood pressure response to air-jet stress during adult life; by contrast, this difference was absent in the presence of much higher chronic elevations in plasma ET-1, observed in ETB-deficient rats **(268)**. Previous findings **(228)**, suggesting decreased functional activity of ET-1 in the presence of chronically elevated plasma levels, provide an explanation for our findings, although further investigation is required

10.1.5. Vagal responses in ETB-deficient rats

As in wild-type rats, vagal withdrawal was evident also in ETB-deficient rats, although the magnitude of this effect differed, as shown by the percent changes in PNSi. The findings of this study shed more light into the interaction between ET-1 and vagal activity, a topic remaining poorly understood. In male Wistar rats dual (ETA and ETB) receptor blockade for 7 days increased sympathetic drive and lowered vagal activity, rather indicating peripheral sites of action in the reflex arc **(264)**. In addition, several pieces of evidence indicate potent interaction between ET-1 and vagal responses at the brain level. Early studies showed a time- and dose-dependent increase in mRNA levels of muscarinic acetylcholine receptors by ET-1 in cultured cerebellar granule cells **(269)**, whereas vagal activation was observed after ET-1 administration either intracisternally **(147)** or selectively in the dorsal vagal complex **(148)** of anesthetized rats. Moreover, low dose intrathecal injection of ET-1 in conscious rats induced

tachycardia; importantly, however, bradycardia was observed at higher dosages in conscious rats, often leading to bradycardic arrest **(262)**.

Interestingly, the effects of ET-1 on vagal responses appear to vary, depending on the type and intensity of AES. Important data on this topic comes from the work by Kurihara et al **(253)**, who examined the role of ET-1 in central vagal responses to emotional stress using wild-type and heterozygous ET-1-knockout mice, which have lower plasma levels of ET-1. The latter strain exhibited diminished autonomic responses to the much higher intensity intruder stress but responded to restraint more intensely than wild-type mice. Substantial evidence stems also from a clinical study in patients with chronic stable coronary artery disease; in these patients, AES in the form of anger recall showed close correlation between vagal withdrawal (assessed by HRV) and plasma ET-1 levels **(214)**. Thus, our results, examined in the context of previous data **(253)**, raise the hypothesis that ET-1 may affect responses in various types of emotional stress.

10.1.6. Rhythm disturbances in wild-type and ETB-deficient rats

In keeping with previous findings **(254)**, PVCs were observed in wild-type rats during acute emotional stress. Interestingly, frequent PVCs were observed in this strain also during recovery, coinciding with prolonged vagal withdrawal and enhanced voluntary motion. This finding indicates prolonged anxiety after AES and underscores the need of encompassing long observational periods in rat models of AES. Extended observation increases its translational value, based on the frequent delayed ventricular tachyarrhythmias recorded after AES in clinical and epidemiological studies **(159, 189, 270-272)**.

Contrasting the rhythm disturbances observed in wild-type rats, bradyarrhythmic events, mainly in the form of sinus pauses, were observed in ETB-deficient rats; the number of these episodes increased from baseline, with subsequent decrease during recovery. This finding was unexpected and, to our knowledge, not previously described in rat models of stress. Its

relevance to human pathophysiology is uncertain, although it resembles vagal stimulation and bradycardia during freezing reactions that can lead to neurocardiogenic syncope.

10.1.7. Freezing reactions to acute emotional stress

The observed picture may be considered representative of a freezing reaction in ETB-deficient rats, a view supported by the low voluntary activity post-AES in these animals. Indeed, freezing reactions in animal models are characterized by a motionless posture after a threat of moderate intensity causing fear **(273)**. Such complex responses are likely accompanied by accentuated sympatho-vagal interaction, leading to vagal dominance, 'fear bradycardia' and syncope **(261)**.

10.1.8. Neurocardiogenic syncope

Vasovagal syncope consists of excessive vagal stimulation leading to bradycardia and/or hypotension in response to various stimuli, including AES. The link between high baseline ET-1 levels and vasovagal syncope was suggested in a small series of pediatric **(216)** and adult **(274)** subjects, whereas further evidence was provided by gene studies in patients with vasovagal syncope **(275, 276)**. Interestingly, such responses are clinically observed in certain personality traits with depressive characteristics **(277)** that have been linked to high ET-1 levels in population studies **(278)**. It should be noted that the pattern of bradycardia in this study recordings (Figure 25) closely resembles the responses observed during the provocation of neurocardiogenic syncope by head-up tilting in clinical practice. The intriguing hypothesis of ET-1 mediating vagal responses in neurocardiogenic syncope merits further study.

10.2. SECOND PART MAIN FINDINGS

The experiments of the second part of the study demonstrate stimulation of sympathetic nervous system in response to acute myocardial infarction in the wild-type rats. On the contrary, sympathetic stimulation in the absence but not in the presence of acute emotional stress in the ETB deficient rats, is observed. This evidence is in line with the first part of the study results and suggest that autonomic responses were blunted in ETB-deficient rats, especially regarding the sympathetic arm, in the presence of acute emotional stress. More importantly, the blunted sympathetic response in ETB-deficient rats was reflected in frequent bradyarrhythmias after the acute myocardial infarction, contrasting the frequent tachyarrhythmic episodes observed in wild-type rats.

10.2.1. Infarct size and mortality

In keeping with previous findings, the ligation of the left coronary artery in Wistar rats seems to be a reproducible model, with little variation in the induced infarct size **(220)**. Thus, it is safe to conclude that the results of this study are comparable. Moreover, the differences observed in arrhythmogenesis of the two rat strains did not translate into difference in the survival of the experimental animals.

10.2.2. Sympathetic responses post-MI in wild-type rats

Activation of the sympathetic arm was observed in the recovery period following the acute myocardial infarction in wild-type rats. Thus, in keeping with previous findings in rats **(70)** and humans **(44)**, the findings of this study underline the role of sympathetic activation after acute MI. Indeed, the role of sympathetic activation is thought to be critical in the maintenance of an adequate cardiac output in response to acute myocardial infarction. Moreover, since not significant differences were observed in the autonomic responses of the

wild-type rats after myocardial infraction between the presence or the absence of acute emotional stress, it is safe to conclude that acute stress does not affect the outcome of the autonomic responses is the setting of myocardial infarction.

10.2.3. Sympathetic responses post-MI in ETB-deficient rats

An important finding of the present study was the different responses to acute myocardial infarction in the ETB-deficient rats, thereby reiterating the role of ET-1 in this setting. This strain displayed a trend of sympathetic enhancement in response to acute myocardial infarction throughout the observation period in the absence of stress, but the difference in comparison to the baseline value was not of marginal significance. This result seems to be in line with the markedly enhanced sympathetic activation in this rat-strain, observed during global **(266)** or regional **(126)** ischemia, or in the setting of heart failure due to pressure overload **(267)**.

In the presence of acute emotional stress, sympathetic nervous system activity remained stable post-MI in the ETB deficient rats. This result is in line with the previous part of the study, in which enhanced baseline levels of ET-1 blunted the sympathetic response to acute emotional stress. In line with the previous part of this study is also the motor activity of the ETB deficient rats in their cage during recovery, after acute emotional stress and acute myocardial infarction. Previous findings **(228)**, suggesting decreased functional activity of ET-1 in the presence of chronically elevated plasma levels, provide an explanation for our findings, although further investigation is required.

10.2.4. Rhythm disturbances in wild-type and ETB-deficient rats post-MI

The unrestrained rat's continuous ECG may now be recorded for longer periods of time thanks to small telemetry devices. This study provides new knowledge about the arrhythmia

profile during the acute phase of MI by describing the uninterrupted time course of tachy- and bradyarrhythmias in conscious, unrestricted rats during the first eight hours following acute coronary occlusion.

In both wild-type and the rats with increased plasma ET-1 (ETB deficient rats), after a baseline recording in which ventricular ectopic beats were extremely rare, a sudden change in arrhythmia frequency began within minutes after the left coronary artery occlusion. An eight-hour arrhythmias profile was recorded, combining the two distinct arrhythmogenic periods. Within this period, severe and frequent episodes of VTs/VFs, premature ventricular contractions (PVCs), couplets, triplets and bradyarrhythmic events; both sinus pauses and atrio-ventricular blocks, occurred. This arrhythmogenic profile following myocardial infarction has long been described in the rat myocardial infarction model **(83)**.

In wild-type rats the incidence and total duration of VTs/VFs was elevated in comparison to the ETB deficient rats in the absence of stress. This result is not in line with previous recordings of VTs/VFs, where no difference was observed in the number of this type of arrhythmias, while in this study they reported longer duration of each VT/VF episode in the ETB deficient rats **(72)**. Additionally, wild-type rats had greater numbers of PVCs, couplets and triplets than the ETB deficient rats, in the absence of stress, which partially explains the more frequent incidents of VTs/VFs since PVCs are known to increase the risk of ventricular tachyarrhythmias **(279)**.

Contrasting the rhythm disturbances observed in wild-type rats, bradyarrhythmic events, mainly in the form of sinus pauses, were observed more frequently in ETB-deficient rats; This finding was unexpected and, not previously described in the ETB deficient rat model of acute myocardial infarction. Its relevance to human pathophysiology is uncertain.

Since no differences in the arrhythmogenesis were observed between the rat groups with the presence or the absence of acute emotional stress, it is safe to conclude that acute emotional stress does not affect the outcome of myocardial infarction on arrhythmogenesis.

10.3. STRENGHS AND LIMITATIONS

In this study, the effects of ET-1 on AES were examined in a previously characterized rat-model displaying chronically elevated ET-1 levels, thereby circumventing the disadvantages associated with exogenous ET-1 administration. All recordings were continuously performed in conscious rats and the analysis was based on well-established methods. Thus, our experiments permit the assessment of sympathetic and vagal responses, as well as rhythm disturbances, over a prolonged observational period. Although ventricular tachyarrhythmias resulting from excessive sympathetic activation have been at the center of previous research efforts, our paper draws attention to bradyarrhythmic events as important rhythm disturbances in response to AES. Despite these merits, two limitations should be acknowledged: First, the experiments included only one AES protocol, though to be specific for investigating mostly emotions of fright or fear. However, the rat responds differently to various stressors, such as social defeat or electric foot shock; hence, the results of this study do not apply to other common conditions, such as anger. Second, in the determination of the sympathetic nervous system activation, it was not possible to measure the levels of catecholamines in the blood and in the myocardium. However, previous data demonstrate very good correlation between heart rate variability parameters and catecholamine measurement. Another limitation that has to be noted is the combination of the two distinct arrhythmogenic periods post-MI, since it has been found that those two periods exhibit different arrhythmogenic characteristics.

10.4. SUGESTIONS FOR FUTURE RESEARCH

The knowledge and experience gained throughout the study process led to new research ideas, as summarized below:

- The role of ET-1 to other common stressors, such as anger or sadness should be investigated, since this study presents only one AES protocol, though to be specific for investigating mostly emotions of fright or fear.

- Chronic stress in the form of anxiety disorders have emerged as an epidemic for contemporary society. In addition to the compromise in the quality of life evoked by these disorders, there is evidence for a causative association between chronic psycho-emotional stress and cardiovascular disease. Several pieces of evidence indicate that chronic emotional stress may be also linked with fatal ventricular tachyarrhythmias. The ET-1 role on the pathophysiology of this type of stress should also be investigated.

- The two distinct arrhythmogenic periods post-myocardial infarction; phase I and phase II, should be investigated separately. This study may shed light into the mechanisms underlying the role of ET-1 in presence of bradyarrhythmias.

D. CONCLUSIONS

Sympathetic activation, prolonged vagal withdrawal and frequent PVCs occur in response to acute emotional stress (AES) in rats under conditions of normal myocardium. ETB-deficient rats, a strain with previously demonstrated high plasma ET-1 levels, display markedly blunted responses to AES. Although both autonomic arms are affected in this strain, prolonged low sympathetic activity yields vagal dominance mainly during recovery, associated with low voluntary activity and bradycardia. ET-1 seems to have a regulatory role in the autonomic responses to acute emotional stress, with a more significant effect on the vagal arm of the autonomic nervous system. The effect of ET-1, during and after myocardial infarction, on the vagal reflex is extended, but not its effect on the sympathetic arm of the autonomic nervous system. The findings of this research strengthen the link between ET-1 and autonomic responses to AES and myocardial infarction and provide further insights in the pathophysiology of stress-induced tachy- and bradyarrhythmias. More studies are needed to confirm whether the acute emotional stress affect the autonomic responses elicited after permanent ischemia.

ΠΕΡΙΛΗΨΗ

Ένα πανταχού παρόν πεπτίδιο, η ενδοθηλίνη-1 (ET-1) είναι επί του παρόντος υπό διερεύνηση ως ρυθμιστικός παράγοντας των αυτόνομων αποκρίσεων στο οξύ συναισθηματικό στρες. Τα βασικά επίπεδα της ενδοθηλίνης στο πλάσμα αλλάζουν τις αποκρίσεις στην πίεση του αίματος, αλλά παραμένει ασαφές εάν επηρεάζουν την αυτόνομη δραστηριότητα και η αρρυθμιογένεση. Επιπλέον, η ET-1 μπορεί να έχει ρυθμιστική δράση και στις αυτόνομες αποκρίσεις του οξέος εμφράγματος του μυοκαρδίου, που συχνά ακολουθεί κάποιο περιστατικό οξέος συναισθηματικού στρες, αλλά και στην προκαλούμενη από έμφραγμα αρρυθμιογένεση. Η αξία αυτού του ευρήματος μετά από ισχαιμία είναι επίσης ασαφής. Στην παρούσα διατριβή μελετήθηκαν συμπαθητικοί και παρασυμπαθητικοί δείκτες (που προέρχονται από ανάλυση μεταβλητότητας καρδιακού ρυθμού), οι διαταραχές του καρδιακού ρυθμού, η εκούσια κίνηση και η πίεση του αίματος μετά από οξύ συναισθηματικό στρες και μετά από μόνιμη απολίνωση της αριστερής στεφανιαίας αρτηρίας.

Η μελέτη έγινε σε επίμυες που είχαν τις αισθήσεις τους, με εμφυτεύσιμους πομπούς τηλεμετρίας. Συγκρίθηκαν δύο στελέχη επίμυων, συγκεκριμένα, επίμυες άγριου τύπου και με επίμυες με έλλειψη του ETB υποδοχέα της ενδοθηλίνης, με τους τελευταίους να παρουσιάζουν αυξημένη ET-1 πλάσματος.

Ως αποτέλεσμα του οξέος συναισθηματικού στρες, δεν υπήρχαν εμφανείς διαφορές στον καρδιακό ρυθμό ή την πίεση του αίματος, αλλά οι συμπαθητικές αποκρίσεις ήταν μειωμένες σε επίμυες με ανεπάρκεια του ETB, σε αντίθεση με την άμεση ενεργοποίηση που καταγράφηκε στους επίμυες άγριου τύπου. Παρατηρήθηκε απόσυρση του παρασυμπαθητικού νευρικού συστήματος και στα δύο στελέχη κατά την έναρξη του στρες, αλλά η δραστηριότητα του παρασυμπαθητικού αποκαταστάθηκε στους επίμυες με έλλειψη του ETB, συνοδευόμενη από χαμηλή εκούσια κίνηση. Αντικατοπτρίζοντας αυτά τα διακριτά αυτόνομα μοτίβα, καταγράφηκαν συχνές έκτακτες κοιλιακές συστολές στους επίμυες άγριου τύπου, σε αντίθεση με τις φλεμβοκομβικές παύσεις που εμφανίζονταν στους επίμυες με έλλειψη ETB.

Φαίνεται πως το οξύ συναισθηματικό στρες δεν υπεισέρχεται σαν παράγοντας στην έκβαση του οξέος εμφράγματος του μυοκαρδίου. Παρόλα αυτά, η ενδοθηλίνη-1 επηρεάζει την αρρυθμιογένεση μετά το οξύ έμφραγμα του μυοκαρδίου, καθώς παρουσία αυξημένων επιπέδων της στο πλάσμα επίμυων τα επεισόδια και η συνολική διάρκεια των ταχυκαρδιών είναι σημαντικά μειωμένα, ενώ τα επεισόδια βραδυαρρυθμιών είναι σημαντικά πιο συχνά από ότι στους επίμυες αγρίου τύπου. Τα αποτελέσματά, της μελέτης αυτής, υποδεικνύουν ότι τα χρόνια αυξημένα επίπεδα ET-1 στο πλάσμα μειώνουν τις αυτόνομες αποκρίσεις στο οξύ συναισθηματικό στρες και το οξύ έμφραγμα του μυοκαρδίου, με επακόλουθη κυριαρχία του παρασυμπαθητικού νευρικού συστήματος, που οδηγεί σε βραδυαρρυθμίες. Τα ευρήματά αυτά παρέχουν περαιτέρω πληροφορίες για την παθοφυσιολογία των ταχυαρρυθμιών που προκαλούνται από το στρες, το οξύ έμφραγμα του μυοκαρδίου αλλά και τη νευροκαρδιογενή συγκοπή.

SUMMARY

"Effect of endothelin receptors on sympathetic stimulation. Experimental study."

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An ubiquitous peptide, endothelin-1 (ET-1) is currently under investigation as a modulatory factor of autonomic responses to acute emotional stress. Baseline plasma endothelin levels alter blood pressure responses, but whether they affect autonomic activity and arrhythmogenesis remains unclear. In addition, ET-1 may also have a modulatory role on the autonomic responses of acute myocardial infarction, which often follows an episode of acute emotional stress. In addition, it may be involved on infarct-induced arrhythmogenesis. The importance of this finding after ischemia is also unclear. In this thesis, sympathetic and parasympathetic indices (derived from heart rate variability analysis), rhythm disturbances, voluntary motion and blood pressure were studied after acute emotional stress and after permanent ligation of the left coronary artery.

The study was conducted in conscious rats with implantable telemetry transmitters (Holter). Two strains of rats were compared, namely, wild-type and rats deficient for the endothelin receptor ETB, the latter displaying elevated plasma ET-1.

As a result of acute emotional stress, no differences in heart rate or blood pressure were evident, but sympathetic responses were blunted in ETB-deficient rats, in contrast to the immediate activation recorded in wild-type rats. Vagal withdrawal was observed in both systems at the onset of stress, but vagal activity was restored in ETB-deficient rats, accompanied by low voluntary motion. Reflecting such distinct autonomic patterns, frequent premature ventricular contractions were recorded in wild-type rats, in contrast to sinus pauses in ETB-deficient rats.

It seems that acute emotional stress does not play a role in the outcome of acute myocardial infarction. Nevertheless, endothelin-1 affects arrhythmogenesis after acute myocardial infarction, as in the presence of increased levels in the plasma of rats, the episodes and the total duration of tachycardias are significantly reduced, while episodes of bradyarrhythmias are significantly more frequent than in wild-type rats. The results of this study indicate that chronically elevated plasma ET-1 levels blunt autonomic responses to acute emotional stress and acute myocardial infarction, with subsequent predominance of the parasympathetic nervous system, leading to bradyarrhythmias. These findings provide further insight into the pathophysiology of stress-induced tachyarrhythmias, acute myocardial infarction, and neurocardiogenic syncope.

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