universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

Effects of anti-anginal drugs ranolazine, nicorandil and trimetazidine on inflammation and atherosclerotic plaque progression

verfasst von / submitted by

Zorica Krivaja

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2018 / Vienna, 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Ao.Univ.-Prof. Dr. Johann Wojta

Table of Contents

Acknowledgments

First of all, I would like to thank Prof. Dr. Wojta for agreeing to supervise my work and my thesis, and for the possibility to work in His laboratory. It was an honor to be a part of such successful lab-team. The positive working atmosphere and great communication in the laboratory made the work on this thesis much easier.

I would like to thank Dr. Speidl for letting me in on my first project in clinical research.

Further, I would like to thank all the other colleagues who were always there to help and teach me the lab-work, as well as answer all the question I had. Their help made this work so much easier.

I thank my mother for always being there for me, for believing in me, even when I doubted myself. Her love and support made it possible for me to achieve my goals so far and kept me always going further and wanting more.

1.Summary

1.1. Abstract

Background. Angina pectoris is a common symptom of ischaemic heart disease (IHD). In this condition exists a disproportion between oxygen demand and oxygen consumption caused by narrowing of coronary arteries. The most common pathological reason for these narrowing is atherosclerosis. Atherosclerosis is today considered to be an inflammatory disease. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and tissue factor (TF) play an important role in this inflammatory reaction. In addition, cytokines and chemokines lead to macrophage and T-lymphocytes accumulation within the vessel wall. The aim of this thesis was to analyze the potential anti-inflammatory and anti-atherosclerotic effect of anti-anginal drugs ranolazine, nicorandil and trimetazidine.

Methods. Human umbilical vein endothelial cells (HUVECs) were treated with ranolazine, trimetazidine and nicorandil. Additionally, HUVEC were treated with or without interleukin (IL)-1ß. Specific mRNA levels for target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by real time polymerase chain reaction (RealTime PCR). For the measurement of ICAM-1, VCAM-1, E-Selectin and TF surface expression on HUVEC flow cytometry was used. To determine the PMN adhesion to HUVEC, an adhesion assay was performed. In order to exclude any cytotoxic effects of the above mentioned drugs lactat dehydrogenase (LDH) assay was performed.

Results. Ranolazine significantly downregulated Il-1ß induced expression of ICAM-1, VCAM-1, E-selectin and TF on mRNA level. In addition, ranolazine reduced IL-1ß induced surface expression of already ICAM-1, VCAM-1, E-selectin and TF. PMN adhesion to HUVEC was also significantly reduced. Nicorandil showed no effect on IL-1ß induced expression of ICAM-1, VCAM-1, E-selectin and TF and it also did not affect the PMN adhesion to HUVEC. Trimetazidine a showed significant downregulation of VCAM-1 on mRNA levels. However, it also upregulated the expression of ICAM-1, E-selectine and TF on mRNA levels but it had no effect on IL-1ß induced surface expression of these adhesion molecules.

Conclusion. The fact that ranolazine significantly downregulated the expression of adhesions molecules confirms the idea of its anti-inflammatory effects. The administration of ranolazine in angina pectoris patients could be beneficial on several levels. Even though nicorandil showed no significant influence on expression of adhesions molecules and therefore shows no anti-inflammatory effect, its mechanism of action still provides benefits to angina pectoris patients. Trimetazidine influence on expression of adhesions molecules should be further examined.

1.2. Zusammenfassung

Hintergrund. Angina pectoris ist ein Symptom der koronaren Herzkrankheit bzw. Dabei kommt es zu einer Disproportion zwischen Sauerstoffangebot und Sauerstoffverbrauch. Die wichtigste pathologische Ursache dafür ist die Atherosklerose welche eine inflammatorische Erkrankung ist. Zelladhäsionsmoleküle wie intrazelluläres Zelladhäsionsmolekül 1 (ICAM-1), vaskuläres Zelladhäsionsmolekül-1 (VCAM-1), E-selectin und Gewebefaktor (TF) spielen eine große Rolle in der Entzündungsreaktion, genauso wie Zytokine sowie Chemokine, die zu einer Akkumulation von Makrophage und T-Lymphozyten in der Wand der Gefäße führen. Ziel dieser Diplomarbeit war zu überprüfen, ob die Arzneimitteln Ranolazine, Nicorandil und Trimetazidine eine mögliche entzündungshemmende Eigenschaft besitzen.

Methoden. Endothelzellen aus humanen Nalbelschnurvenen (HUVEC) wurden mit den Arzneimitteln Ranolazine, Nicorandil und Trimetazidine inkubiert. Anschließend wurden die HUVEC mit oder ohne Interleukin (IL)-1ß inkubiert. mRNA für ICAM-1, VCAM-1, E-selectin, TF und GAPDH wurden mittels RT-PCR bestimmt. Mittels Durchflusszytometrie wurde die Bestimmung der Zelloberflächen Expression der Adhäsionsmolekülen durchgeführt. Die Adhäsion der neutrophilen Granulozyten an die HUVEC wurde gemessen. Um potentiell toxische Effekte auszuschließen haben wir einen LDH Test durchgeführt.

Resultate. Ranolazine hemmte IL-1ß-induzierte Expression von ICAM-1, VCAM-1, E-selectin und TF signifikant. Die IL-1ß-induzierte Oberflächenexpression der Adhäsionsmoleküle wurde auch deutlich reduziert. Ranolazine hemmte ebenfalls die Adhäsion von neutrophilen Granulozyten. Nicorandil hatte keinen Effekt auf die IL-1ß-induzierte Expression von ICAM-1, VCAM-1, E-selectin und TF gezeigt. Die Adhäsion von neutrophilen Granulozyten wurde nicht beeinflusst.
Trimetazidine hemmte signifikant VCAM-1 Expression. Hingegen wurde die Expression von ICAM-1, E-selectin und TF hochreguliert.

Diskussion. Dadurch, dass Ranolazine die Expression von Adhäsionsmolekülen signifikant herunterreguliert hat, wurde die Idee über die möglichen Enzündungshemmenden Effekte bestätigt. Nicorandil hat keinen enzündungshemmenden Effekt gezeigt, trotzdem ist sein Wirkungsmechanismus sehr effektiv in der Behandlung von Angina pectoris. Der Einfluss von Trimetazidine auf die Enzündungsmediatoren sollte noch weiter erforscht werden.

2. Introduction

2.1. Angina Pectoris

Ischemic heart disease (IHD) also known as coronary artery disease (CAD) includes group of diseases like angina pectoris, myocardial infarction and sudden cardiac death [5]. IHD is the leading cause of death in the United States with angina pectoris as a common symptom of this disease. The American Heart Association (AHA) estimates that 6.8 million Americans suffer from angina and that 400 000 new patients present with stable angina each year. Significantly more women have the condition than men [6, 7]. In this condition exists a disproportion between oxygen demand and oxygen consumption [8]. Leading clinical symptoms are discomfort in the chest, jaw, shoulder, or arms that are induced by physical exertion or emotional stress while some patients do not experience discomfort but complain of breathlessness or tire with activity [9]. Less typically, discomfort may occur in the epigastric area. Pain can also be perceived in back because of the close proximity of the spinal nerve receptors to these areas. The duration of the discomfort is brief, not more than 10 min in the majority of cases, and more commonly even less [10].
Angina limits normal daily activities and has a negative impact on quality of life (QOL). It has been shown that 1 year after coronary revascularization, around one third of patients with angina are not able to return to work [11]. Regardless of revascularization and therapy, up to 26% of patients still experience attacks [12, 13]. While there have been considerable advances in medical management, surgical and percutaneous revascularization strategies, many patients remain symptomatic at substantial personal and societal cost [14].

2.1.1. Stabile angina pectoris

Stabile angina pectoris includes different phases of CAD, with the exception of acute coronary syndromes (ACS) [15]. In addition atherosclerosis is the most common pathological cause of chronic stable angina as well as acute coronary syndromes [16]. Narrowing of 50 % or more in the left main coronary artery and 70 % or more in one or several of the major coronary arteries represent the pathophysiological mechanism underlying stable angina pectoris, causing exercise- and stress-related chest symptoms [15]. Estimated annual mortality rates range from 1.2 to 2.4 %, with an annual incidence of cardiac death between 0.6 and 1.4 % [17-19].
The aim of stable angina management is the symptomatic relief and the secondary prevention [16]. For patients with stable angina, it is also useful to classify the severity of symptoms. (Table 1) [20].

Classification of angina severity according to the Canadian Cardiovascular Society

Class	Level of symptoms
Class I	'Ordinary activity does not cause angina'
	Angina with strenuous or rapid or prolonged exertion only
Class II	'Slight limitation of ordinary activity'
	Angina on walking or climbing stairs rapidly, walking uphill or exertion after meals, in cold weather, when under emotional stress, or only during the first few hours after awakening
Class III	'Marked limitation of ordinary physical activity'
	Angina on walking one or two blocks on the level or one flight of stairs at a normal pace under normal conditions [a]
Class IV	'Inability to carry out any physical activity without discomfort' or 'angina at rest'

[a] Equivalent to 100–200 m.

Table 1. *Canadian Cardiovascular Society Classification of angina pectoris [20]*

2.1.2. Pathogenesis of angina pectoris

Myocardial ischemia can manifest as a discomfort having various characteristics or can remain totally silent [21]. Angina pectoris manifests as stress-induced ischaemic episodes resulting in severe chest pain [22]. Most events that trigger anginal pain do so by changing myocardial oxygen demand. These triggers may be physical, emotional, or metabolic [23].
Among the variety of causes of angina pectoris, two major categories may be recognized: (1) the anatomic, causing arterial obstruction, such as atherosclerosis which is responsible for about 90% of the angina cases, and (2) the functional causes that include aortic valvular disease and functionally related conditions, thyroid disease, and pulmonary hypertension [24].
Myocardial ischaemia occurs when there is a deficit between the myocardial oxygen delivery that is required for normal heart rate, and the actual rate of oxygen delivery [25] and it is caused by narrowing of cardial arteries.
The primary result of ischaemia, next to the reduced oxygen consumption, is reduction of adenosine triphosphate (ATP) formation in the mitochondria. Normally in healthy heart the energy for ATP resynthesis in the mitochondria comes from the combustion of fatty acids and to a minor extent from carbohydrate. Also, under normal conditions the heart consumes lactate. With ischaemia there is reduced aerobic formation of ATP in the mitochondria, which

stimulates non-oxidative anaerobic glycolysis and a switch from myocardial lactate uptake to lactate production. As result, lactate accumulates, and it comes to cell acidosis [26-29].
Low intracellular pH has numerous negative effects on the ability of the cardiomyocyte to use the energy released from the hydrolysis of ATP to maintain Ca^{2+} homeostasis and perform contractile work. The Ca^{2+} concentration needed for a given force generation is larger at lower pH. The ATP requirement by the sarcoplasmic reticulum Ca^{2+} pump is higher when pH is decreased [30]. Therefore, when pH is low, more energy released from ATP breakdown goes to the work for regulating Ca^{2+} content in the cytosol, and less is available for contractile work.
Also, ischaemia leads to tissue hypoxia and cell necrosis. A cytokine cascade is being initiated, in the first line by IL-1ß, causing activation of the microvascular endothelium. Once activated, endothelial cells express selectins, such as E-selectin, that results in attachment and rolling of leukocytes. Adhesion molecules like ICAM-1 and VCAM-1 are also being expressed, causing so adhesion and migration of PMN and leading to further tissue damage [31].

2.1.3. Treatment and diagnosis

The description of symptoms, described by patients and recorded by doctors, remains a cornerstone of diagnosis [32]. Angina pectoris must be differentiated from non-atherosclerotic chest pain. Arteriography should be performed when the diagnosis is in doubt or when the stable form becomes unstable [33].
Angina can also serve as an index for detecting families with high risk of CAD, in whom early application of primary prevention may afford a more promising outlook [34].
Many patients with stable angina are treated with more than 1 class of anti-anginal drugs. In practice it is common to encounter patients taking 2 and, at times, 3 different types of anti-anginal therapy [35]. Optimal medical therapy is a combination of antianginal/antiischemic drugs and disease modifying agents, such as nitrates, beta-blockers (metoprolol, propranolol), calcium channel blockers (diltiazem, nifedipine) antiplatelets, statins and angiotensin converting enzyme (ACE) inhibitors. Beta-blockers, however, are absolutely or relatively contraindicated in patients with asthma, chronic obstructive pulmonary disease (COPD), severe peripheral vascular disease (PVD), and in some patients with diabetes mellitus [14]. Novel classes of treatment with different mechanisms of action have been developed in the last years, including ivabradine, ranolazine, nicorandil and trimetazidine [16]. Ranolazine, nicorandil and trimetazidine action through modulation of myocardial metabolism.
The metabolic approach to treating ischaemic heart disease is not new. In the 1960s and 1970s the use of metabolic therapies for treating ischaemic myocardium has shown that an infusion of glucose, insulin and potassium (GIK) reduced ventricular dysrhythmias [36, 37] and increased survival following a myocardial infarction [38, 39]. During this period the concept was developed, that suppression of circulating plasma non-esterified fatty acids, and thus myocardial fatty acid uptake and oxidation, reduced ischaemic damage and ventricular dysrhythmias during acute myocardial infarction or exercise-induced angina [40-42].

Therefore, anti-ischaemic drugs, lifestyle changes, regular exercise training, patient education and revascularization result in long-term prevention of symptoms [15].
However, the most important consideration in the treatment of angina is protection of coronary blood flow reserve by primary prevention of the atherosclerotic process [34].

2.2. Atherosclerosis

Nowadays, atherosclerosis with its complications and clinical manifestations like ischemic heart disease remain the most common cause of death in the western world [43]. The understanding of atherosclerosis pathogenesis had undergone many changes. Several theories about atherogenesis have been proposed during the last decades, but none could completely explain the process of the pathogenesis of atherosclerosis. In spite of this, the concept that atherosclerosis is a specific form of chronic inflammatory process is now days well accepted. Pathophysiological studies have unraveled the interactions of molecular and cellular elements involved in atherogenesis [44, 45].

2.2.1. Pathogenesis of atherosclerosis

As above mentioned today it is accepted that atherosclerosis is an inflammatory disease characterized by lipid-laden lesions within the arterial wall [46]. The most inner layer of the human arteries wall, the intima, is defined as the region of the arterial wall from and including the endothelial surface at the lumen to the luminal margin of the media. Under normal circumstances the intima does not support adhesion of leukocytes and promotes fibrinolysis [47]. Oxidative modified low-density lipoproteins (LDL) play an important role in initiating the inflammation. Accumulation of LDL particles within the vessel wall, due to endothelial dysfunction and structural alterations, triggers a strong inflammatory response. High density lipoprotein (HDL) is a protective factor against atherosclerosis because it has a role in the removal of excess cholesterol from peripheral tissues and also inhibits lipoprotein oxidation [48, 49]. LDL particles are then oxidated, which leads to the activation of endothelial cells [49]. Endothelial cells then express pro-inflammatory molecules, including adhesions molecules such as ICAM-1 and VCAM-1. Increased expression of ICAM-1 and VCAM-1 is considered to be responsible for the adhesion to endothelial cells of monocytes and T lymphocytes and for their preferential binding to the arteria surface [50]. Other molecules that seem to influence on the process of inflammation are E-selectine and P-selectine. P- and E-selectine are adhesion molecules meditating the first step in leukocytes extravasation [51]. After adhering to the endothelium and entering the wall, monocytes accumulate within the arterial wall. In the intima of the inflamed arterial wall, monocytes mature into macrophages under the influence of macrophage colony stimulating factor (M-CSF) [52]. The macrophages express scavender receptors (SR) that can take up oxidized LDL. The expression of SRs is regulated by interferon-gamma (IFN- ɣ) and tumor-necrosis factor-alpha (TNF- α). Cytoplasm becomes rich on LDL particles, giving macrophages

appearance like “foam-cells”. These are typical for early atherosclerotic lesions also known as fatty streaks [53] (Figure 1). If the lesions grow into the arterial lumen they can lead to ischemic stroke, transient ischemic attack, critical limb ischemia, abdominal aortic aneurysm and in heart they can lead to myocardial infarction and unstable angina pectoris [54]. Some plaques tent to destabilisation that can lead to plaque rupture.

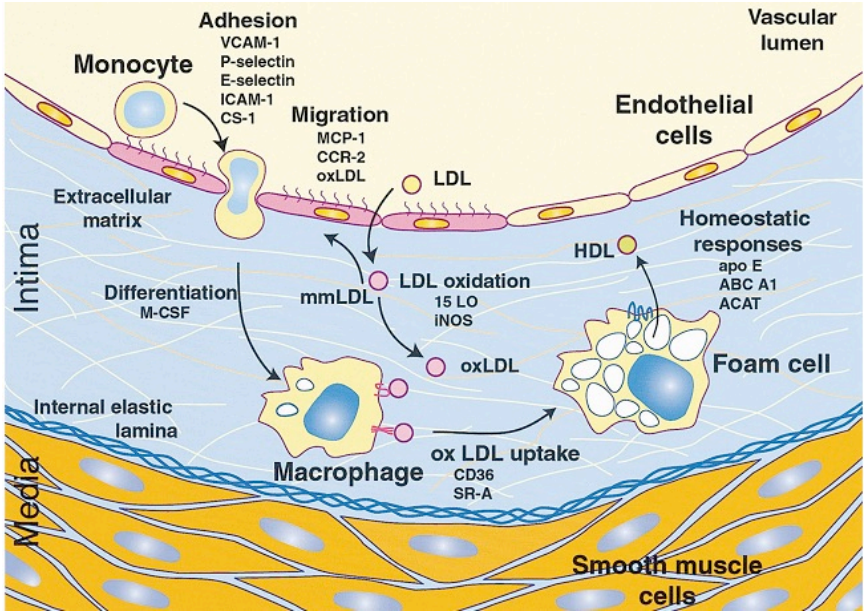

Figure 1. *Initiation of inflammation and foam cell building [2]*

Oxidized LDL particles can also activate Toll-like receptors (TLR) on the surface of macrophages and activate them through that [55]. Once activated they produce pro-inflammatory cytokines such as TNF- α, Interleukin-1, transforming growth factor beta (TGF- ß) and enzymes that destroy arterial extracellular matrix such as metalloproteinases (MMPs). MMPs play later an important role in the plaque destabilisation [56]. On the other hand, accumulated and activated T-lymphocytes produce pro-inflammatory cytokines such as TNF-α and β, IL-1, IFN- ɣ that amplify the inflammatory process [54]. T-cell activation also results in secretion of an immunoregulatory molecule CD40 ligand, CD154. CD154 induces production of MMPs and TF. All together these mechanisms contribute to higher thrombogenicity of atherosclerotic plaque [57-59].

2.3. Ranolazine

IUPAC Name: N-(2,6-dimethylphenyl)-2-[4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]piperazin-1-yl]acetamide

Molecular Formula: $C_{24}H_{33}N_3O_4$

Molecular Weight: 427.545 g/mol

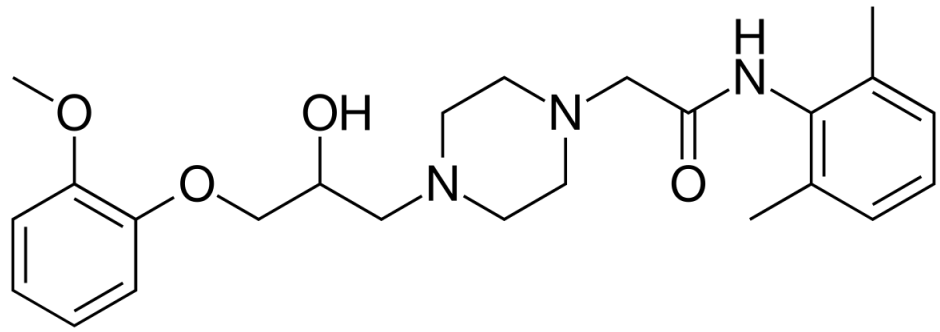

Figure 2. *Chemical structure of ranolazine, a piperazine derivate*

Ranolazine is a new piperazine derivative with anti-ischemic properties attributed to a modulation of myocardial metabolism [60]. It is approved for the treatment of chronic angina pectoris in combination with amlodipine, β-adrenoceptor antagonists (beta-blockers) or nitrates in patients who have not achieved an adequate response with other anti-anginal drugs [61]. Differently from nitrates, β-blockers and Ca^{2+} antagonists that mediate their action through hemodynamic effects, ranolazine has no clinically important effects on blood pressure and heart rate. [62, 63].

2.3.1. Ranolazine mechanism of action

Fatty acids are the main fuel for the healthy heart [1]. However, fatty acids rise precipitously in response to stress, including acute myocardial ischaemia [62]. This elevation of free fatty acids in circulation causes an inefficient use of the already limited oxygen supply. More oxygen is required to phosphorylate ATP during fatty acid oxidation than during carbohydrate oxidation. Therefore, higher free fatty acids concentration suppress the myocardial uptake and oxidation of more oxygen efficient carbohydrates such as glucose and pyruvate, which leads to further oxygen wasting and the accumulation of lactate (Figure 1A) [1].

It appears that ranolazine moves ATP production away from fatty acid oxidation to the adventage of more oxygen efficient carbohydrate oxidation [64]. This shift of ATP production away from fatty acid oxidation seems to reduce the oxygen required to support cardiac work (Figure 1 A and B) [1]. This is one of the possible action mechanisms.

Besides already described mechanism, ranolazine also has an inhibiting effect of the late inward sodium (I_{Na}) current, which reduces calcium overload, and by doing so, it seems to improve left ventricular diastolic dysfunction. By decreasing diastolic tension, ranolazine decreases oxygen consumption. In fact, it has been shown that ranolazine improves left ventricular diastolic function in patients that suffer from ischemic heart disease [65].

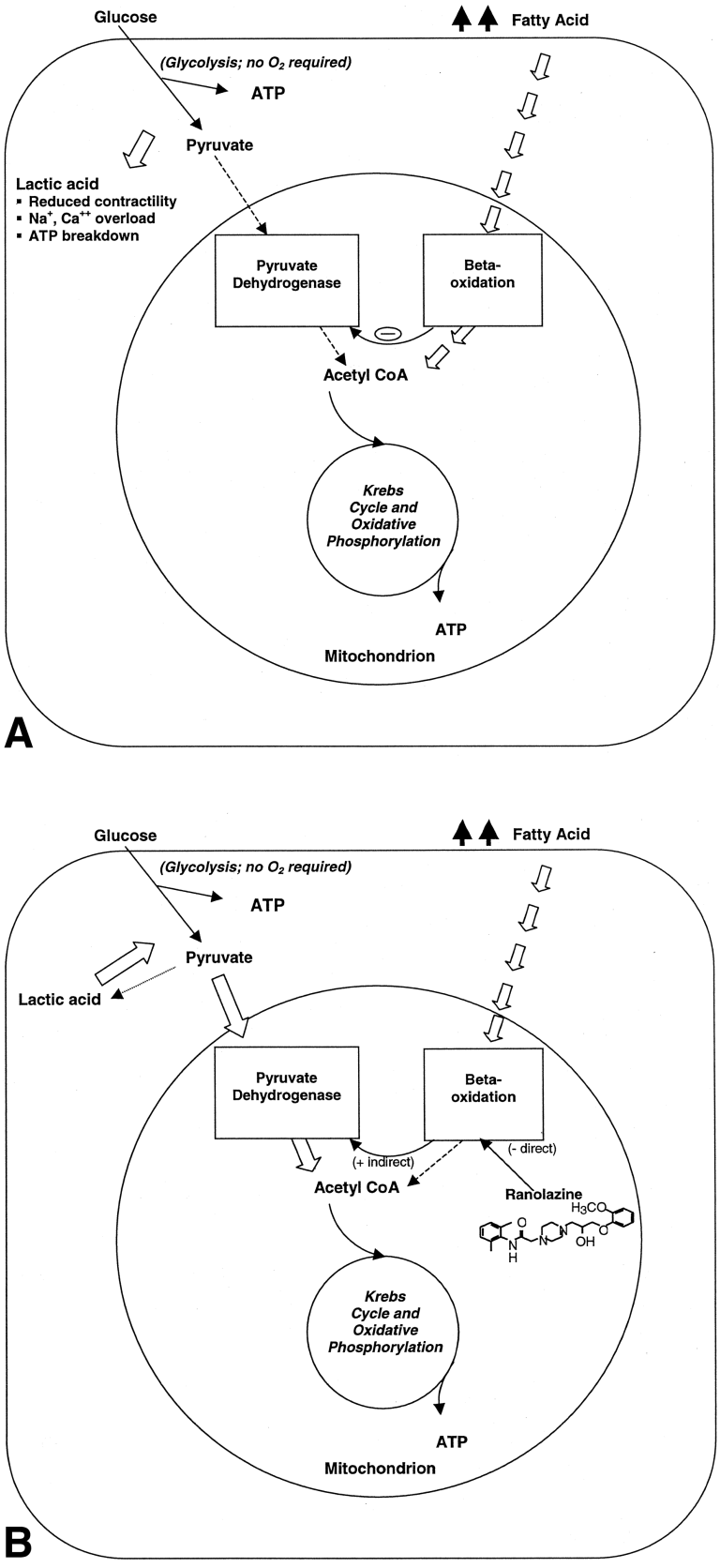

Figure 3. *Regulation of carbohydrate metabolism by fatty acid oxidation. Without ranolazine (A) and with ranolazine (B) [1]*

It is known that ranolazine produces a modest prolongation of the QT interval [66], but besides that ranolazine also produces ion channel effects similar to those observed following amiodarone (reduced I_{Kr}, I_{Ks}, late I_{Na} and I_{Ca}). Ranolazine's actions to suppress early afterdepolarizations (EAD) and reduce transmural dispersion of repolarization (TDR) suggest that, in addition to its anti-anginal effects, the drug may also possess antiarrhythmic activity [67].

2.3.2. Adverse events and metabolism

The drug shows no serious adverse events. All adverse events are mild or moderate in severity. Constipation, headache, asthenia, and nausea were reported as possibly drug related [68].

Metabolism in man was analysed by Liquid chromatography–mass spectrometry LC/MS. The drug is extensively metabolized in the liver by the cytochrome P450 (CYP) 3A and 2D6 enzymes. 5% to 10% is being excreted unchanged by the kidneys. It is an extensive reaction with up to seven primary routes of metabolism. N-dealkylation by hydrolysis at the piperazine ring produces three metabolites, whilst O-demethylation and O-dearylation at the methoxyphenoxy moiety produced a further two compounds. Finally, hydrolysis of the amide group forms one other species. Oxygenation at various points in the molecule produces a further four metabolites. Direct conjugation of ranolazine with glucuronic acid and with an uncharacterized adduct were also identified as a route of elimination. Three major metabolites of ranolazine CVT-2512, CVT-2514, and CVT-2738 are produced by dearylation (CVT-2512), *O*-demethylation (CVT-2514), and *N*-dealkylation (CVT-2738) [69, 70] (Figure 3. *Ranolazine metabolic pathways [4]*)

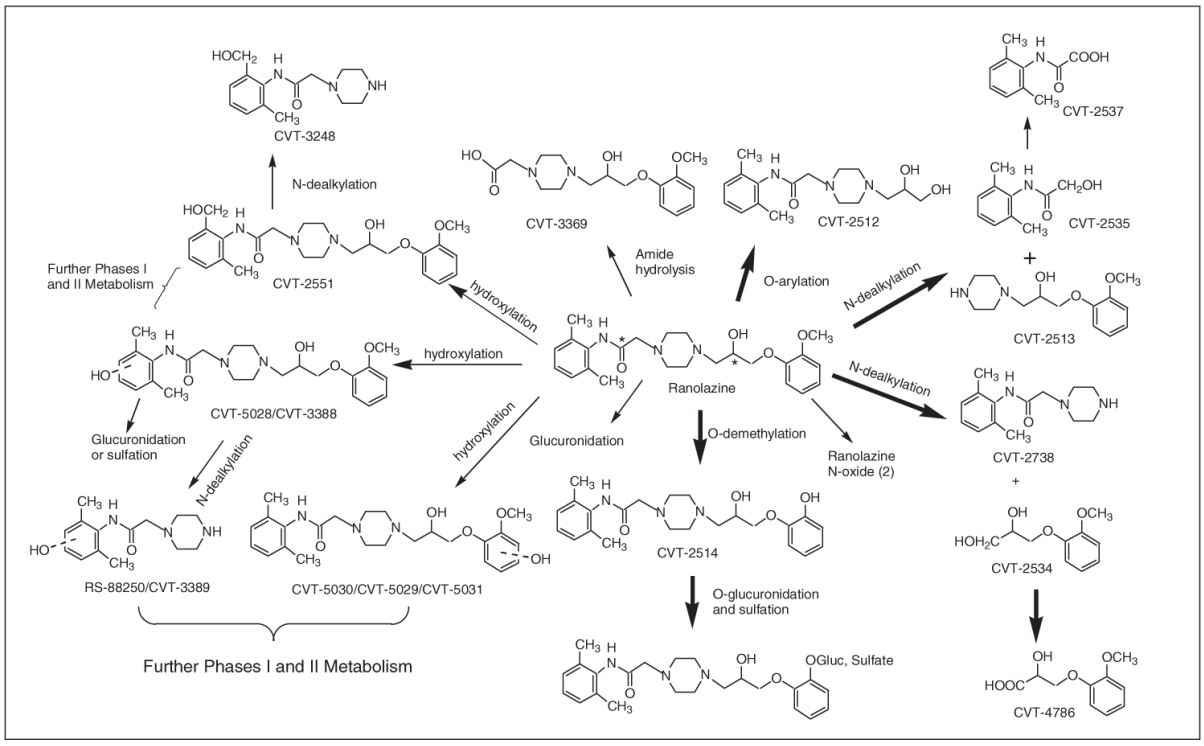

Figure 4. *Ranolazine methabolic pathways[4]*

2.3.3. Interactions

Ranolazine is a weak inhibitor of CYP3A and increases area under the curve (AUC) and maximum plasma concentration ($C_{max)}$ for simvastatin, its metabolites and HMG-CoA reductase inhibitor activity <2-fold. However, simvastatin does not affect ranolazine pharmacokinetics. Digoxin AUC is increased 40–60% by ranolazine through P-glycoprotein inhibition. Ranolazine AUC is increased by CYP3A inhibitors ranging from 1.5-fold for diltiazem 180mg once daily to 3.9-fold for ketoconazole 200mg twice daily. Verapamil increases ranolazine exposure approximately 2-fold. CYP2D6 inhibition has a negligible effect on ranolazine exposure [4, 61].

Multiple trials investigated the efficacy of ranolazine in therapy of stabile angina pectoris.
A randomized, 3-group parallel, double-blind, placebo-controlled trial of 823 eligible adults with symptomatic chronic angina pectoris has shown that twice-daily ranolazine doses ranging from 500 mg to 1500 mg increased exercise capacity and provided additional antianginal relief to symptomatic patients [66].
In another comparative trial of ranolazine versus atenolol it has been shown that ranolazine therapy prolonged exercise duration and decreased exercise-induced ischemia and angina with quantitative effects equal to or greater than those with atenolol [71].
Ranolazine monotherapy in chronic angina patients increased exercise performance throughout its dosing interval at all doses (500, 1000 or 1500 mg) without clinically meaningful hemodynamic effects.

2.4. Nicorandil

IUPAC Name: 2-[(pyridin-3-ylcarbonyl)amino]ethyl nitrate

Molecular Formula: $C_8H_9N_3O_4$

Molecular Weight: 211.175 g/mol

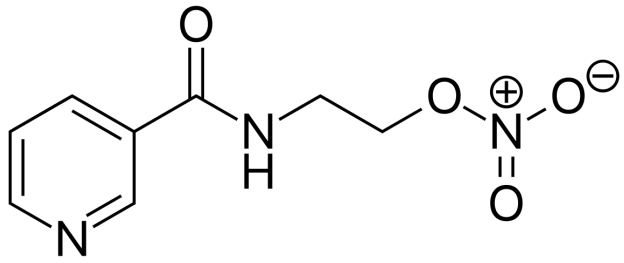

Figure 5. *Chemical structure of nicorandil*

Nicorandil is a 2-nicotinamidethyl nitrate and adenosine triphosphate–sensitive potassium (K_{ATP}) channel opener [72, 73]. It possesses nitrat-like properties [74] and it has been shown

to have an anti-anginal effect comparable with that of β-blockers (atenolol, propranolol) and calcium antagonists (diltiazem) [75].
It has been demonstrated that nicorandil causes increase in oxygen supply without an increase in oxygen demand, as well as favourable endocardial to epicardial blood flow distribution [76, 77]. In addition, hemodynamic studies in patients with coronary heart disease (CHD) have suggested a reduction in pre- and afterload [78]. Therefore, nicorandil affects two of the main hemodynamic determinants of oxygen demand without impairing myocardial contractility or atrioventricular conduction. In addition, its strong spasmolytic activity is of particular interest when dynamic coronary obstruction is considered [79].
Nicorandil is used in therapy of angina pectoris as part of combination therapy, and it is being increasingly used as the first-line treatment in both stable and unstable angina [79-81].

2.4.1. Nicorandil mechanism of action

Nicorandil has two vasodilator mechanisms; ATP-dependent activation of K^+ channels and stimulation of guanylyl cyclase resulting in increases in cyclic guanosine monophosphate (cGMP) [82]. First, nicorandil increases K^+ conductance in the cell membrane, resulting in K^+ outflow from the cell and causing membrane hyperpolarization [83]. It increases cellular levels of cGMP [84] and these both actions cause vasorelaxation (Figure 5). Since nicorandil has a terminal NO_2 group [85], his nitrate-like mechanism dilates epicardial coronary arteries [86] and that leads to an increase of the blood supply to the ischemic region of the heart.
Further, it has been shown that nicorandil opens K_{ATP} channels in ischemic cardiomyocytes [87] and there are indications that the mitochondrial K_{ATP} channels are actually more involved in the protection of ischemic conditioning, than sarcolemmal [88, 89], possibly by reducing the mitochondrial membrane potential [90, 91].
Also it has been suggested that nicorandil might inhibit calcium mobilization from intracellular stores and/or calcium entry into the cell [92]. Nicorandil attenuates norepinephrine-induced sustained contraction, possibly by inhibiting the Ca^{2+} influx through receptor-linked Ca^{2+} channels and also the transient contraction by membrane hyperpolarization [93]. Furthermore, nicorandil suppresses the increase in the free Ca^{2+} concentration within the cell, possibly by stimulating (Ca^{2+} + Mg^{2+})-dependent ATPase and by doing so relaxes vascular smooth muscle [94].

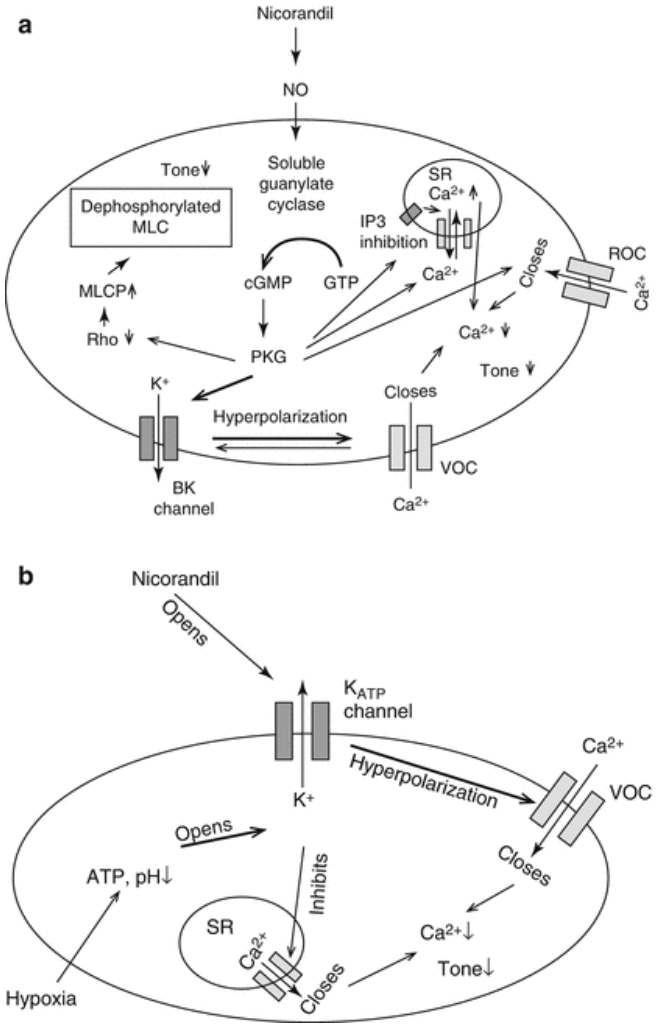

Figure 6. *Molecular actions of nicorandil, showing activation of both cGMP (A) and K+ ATP (B) signaling pathways.* *(Figure adapted and taken from Basicmedical Key)*

2.4.2. Adverse events and metabolism

Common adverse effects include back pain, chest pain, feeling of weakness. Not so rare are hypotension, increased heart rate in higher doses, palpitations and flush may be observed. As a consequence of vasodilatation headaches may arise.

Nicorandil is metabolized extensively. Metabolic studies show that the main biotransformation pathways are denitration and then introduction into the nicotinamide metabolism. In the presence of a NADPH-generating system, nicorandil is being metabolized to its main denitrated metabolite 2-nicotinamidoethanol (SG-86), then releasing NO. Glutathione-S-transferases (GSTs) are not primarily involved in the conversion of nicorandil to SG-86 [95].
The major route of elimination is the kidney: Less than 2% of the dose is excreted through the biliary route. As a consequence, the parent drug is excreted poorly in urine (very low

renal clearance), whereas its main metabolite SG-86 is the major nicorandil-related compound excreted in urine. However, unchanged nicorandil and denitrated metabolite excreted into the urine represent only about 1 and 4% of the dose [96, 97].

2.4.3. Interactions

Care should be taken in clinical practice when prescribing combinations of K_{ATP} channel openers like nicorandil and sulfonylureas. Drugs like glibenclamide or glimpiride, which target SUR1 and SUR2-type K_{ATP} channels, may impair the clinical effectiveness of nicorandil [98]. On the other hand, gliclazide, which specifically blocks SUR1-type K_{ATP} channels, does not affect nicorandil activity. [99]
Also, combination of sildenafil with nicorandil, as well as with nitroglycerin, potentiates the hypotensive response by increase of vasodilatation. Synergism of vasodilation may be linked with NO action, but not with K_{ATP}channel-activation [100].

In a trial with 11 patients with stabile effort angina it has been shown that nicorandil (10mg or 20mg) prolonged the duration of exercise and delayed the onset of ischemic ST depression compared to placebo. Nicorandil also increased the pressure-rate product at the time of angina compared with placebo. These results indicate that nicorandil should be considered for the clinical treatment of effort angina [72].
Also, oral administration of nicorandil may reduce cardiac death and improve the survival of hemodialysis patients after coronary revascularization [73].
When added to standard antianginal treatment in coronary heart disease CHD patients with stable angina, nicorandil significantly reduces the number of ischemic attacks [101].

2.5. Trimetazidine

IUPAC Name: 1-(2,3,4-trimethoxybenzyl)piperazine

Molecular Formula: $C_{14}H_{22}N_2O_3$

Molecular Wight: 266.336 g/mol

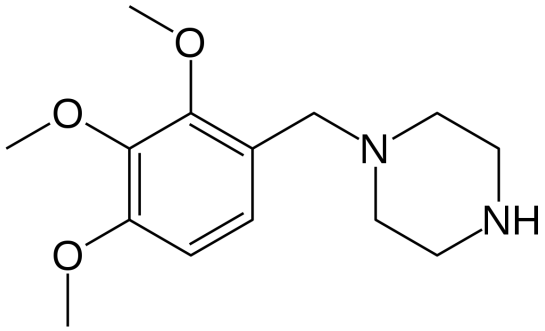

Figure 7. *Chemical structure of trimetazidine*

Trimetazidine is a 1-(2,3,4 trimethoxybenzyl)piperazine dihydrochlore salt (2HCl) that displays anti-ischeamic effects without inducing any significant hemodynamic changes [102]. An unchanged rate x pressure product at rest and at peak exercise, suggest that trimetazidine may exert its anti-ischaemic effect at the cellular level [103, 104].

2.5.1. Trimetazidine mechanism of action

The result of myocardial ischaemia is reduced oxygen consumption and ATP formation in the mitochondria, accelerated anaerobic glycolysis and lactate accumulation. Fatty acids are the primary mitochondrial substrate during myocardial ischaemia. However, during an ischaemia fatty acids reduce the oxidation of carbohydrate and initiate the transformation of pyruvate to lactate, causing lactate accumulation and cell acidosis. The partially inhibition of myocardial fatty acid oxidation increases carbohydrate oxidation, which results in reduced lactate production and a higher cell pH during ischaemia and is an effective treatment for chronic stable angina pectoris [3].

Despite efficacy being equivalent to that of beta-blockers and calcium antagonists [22], studies proved that trimetazidine has no effect on contractility, heart rate, and coronary flow which suggested that it played its effects by improving myocardial energy metabolism [105-108]. The drug acts via partial inhibition of myocardial fatty acid oxidation [109] (Figure 7 [3]). Trimetazidine reduces the long-chain activity of the enzyme acetyl-CoA C-acyltransferase (3-KAT). 3-KAT catalyses the terminal reaction of fatty acid β-oxidation, using long-chain 3-ketoacyl-CoA as a substrate and producing acetyl-CoA. However, trimetazidine does not inhibit the short or medium chain activity of 3-KAT. This suggests that trimetazidine displays its effects trough inhibition of 3-KAT to reduce the NADH/NAD+ and acetyl-CoA/free CoA ratios in the mitochondrial matrix. Trimetazidine significantly increases the rate of glucose oxidation [110, 111].

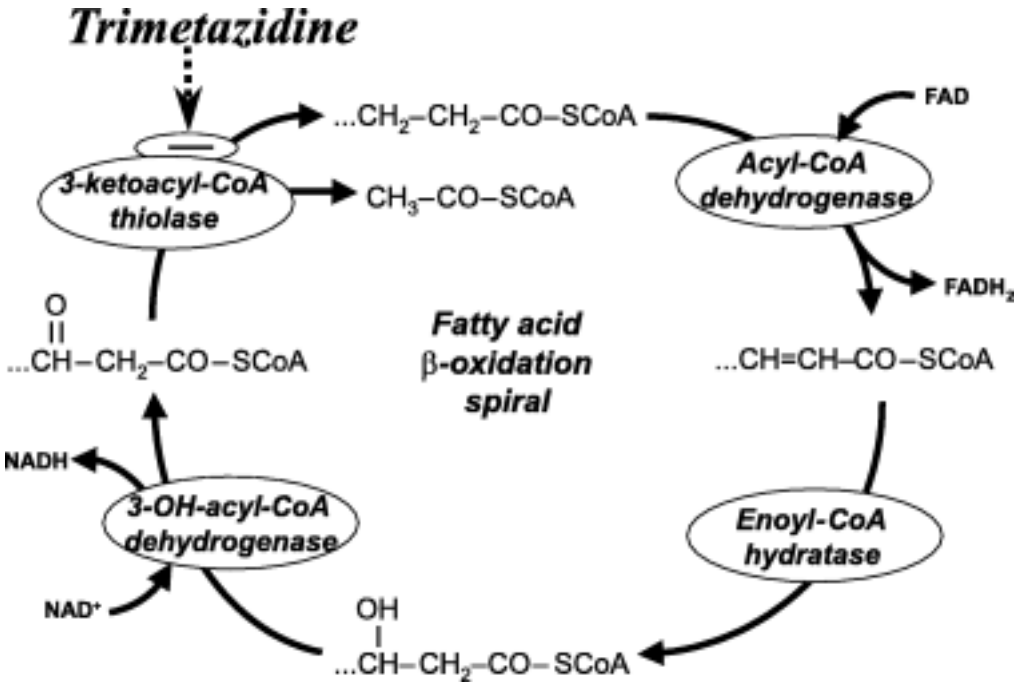

Figure 8. *Trimetazidine inhibits the long-chain activity of 3-KAT [3]*

In the study by Iskesen et al, trimetazidine increased the level of superoxide dismutase and glutathione peroxidase. Both enzymes are part of the endogenous antioxidant enzyme systems. This endogenous antioxidant enzyme system is responsible for limiting intracellular accumulation of oxygen radicals during normal aerobic metabolism. Trimetazidine reduces membrane damage produced by reactive oxygen species and it protects tissue from free radicals through its antioxidant effects [112].

2.5.2. Adverse events and metabolism

Adverse effects of trimetazidine are mild and infrequent [22]. In controlled studies, adverse effects correlated with trimetazidine therapy were gastric or esophageal burning, muscular cramps, dizziness, depression, sedation, palpitations, visual disturbances, anorexia, and hyperorexia [113]. Also, concerns arose regarding the safety of trimetazidine-containing medicines due to numerous reports of Parkinson syndrome and other motor disorders such as tremor, muscle rigidity, and walking disorders and restless legs syndrome [114]. The symptoms were observed in patients with no history of Parkinson syndrome. However, their symptoms resolved once therapy with trimetazidine was stopped [113].
Majority of the drug excreted in the urine. In addition to the parent drug, 10 metabolites were detected in urine in concentrations ranging from 0.01% to 1.4% of the dose [115].

2.5.3. Interactions

Addition of trimetazidine to metoprolol therapy produced significant improvements in exercise stress tests and the symptoms of angina relative to metoprolol alone [116].
In European study (Trimetazidine European Multicenter Study, TEMS) involving 149 patients, trimetazidine (20 mg t.i.d.) was compared with propranolol (40 mg t.i.d.) in patients with stable angina pectoris and documented significant coronary artery stenoses. Both drugs equally reduced the number of anginal attacks, while exercise duration was increased by both treatments. However, in contrast with propranolol trimetazidine did not alter the rate pressure product. In patients already treated with nifedipine or beta-blockers, the addition of trimetazidine (20 mg t.i.d.) was able to reduce the number and the duration of anginal attacks and improved also the exercise capacity [117].
Another study showed that a single dose of 60 mg/d trimetazidine, improved exercise capacity in patients with angina pectoris, resulting in increase of the exercise duration and total work performed. These effects occurred without any noticeable chronotropic or vasomotor effect [104].

3. Aims

The aim of this study was to examine whether the anti-anginal drugs ranolazine, nicorandil and trimetazidine have anti-inflammatory and anti-atherosclerotic effects on human endothelial cells *in vitro* and could thereby modulate the inflammatory process during atherogenesis and could possibly protect from thrombus-formation after erosion or rupture of coronary plaques.

Therefore we investigated whether the treatment of human umbilical vein endothelial cells (HUVEC) with or without above mentioned anti-anginal drugs in combination with the inflammatory cytokine IL-1ß has an effect on expression of adhesion molecules and adhesion of PMN to endothelial cells.

4. Materials and Methods

4.1. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were used for all experiments. HUVECs were isolated from fresh human umbilical cord segments, obtained under sterile conditions. Into the lumen of the umbilical cord vessel vein an adaptor was inserted and clamped. A 20 ml syringe containing Hank's Balanced Salt Solution (HBSS) was attached to the adaptor and the vessel was washed. Another 10 ml syringe with collagenase solution (collagenase typ IV (Sigma), 2mg/ml in HBSS modified) was connected to the adaptor and the vessel was filled with collagenase solution until it leaked at the other end of the vessel. The other end of the vessel was clamped with a hemostat and the vessel was filled. After 45 minutes of incubation at room temperature the hemostat was opened and the perfusate was collected in a centrifuge tube. This was centrifuged for 5 minutes at 1500rpm. The supernatant was carefully removed, and the pellet was resuspenden in minimum essential medium (M199, Sigma) containing 20% fetal calf serum (FSC, Biochrom), antibiotics (PSFG: Penicillin, Streptomycin, Fluconazol, L- Glutamine; all from Cambrex, Esat Rutherford, NJ, USA), 5 U/ml heparin and 5 µg/ml endothelial cell growth supplement (ECGS). Resuspended cells were transferred into an appropriate cell culture flask and coated with 1% gelatine. The flasks were stored at 37°C in a humidified incubator (Thermo Scientific Heraeus Cytoperm2) consisting 95% air and 5% CO_2. The cells between passages 2 and 5 were used.

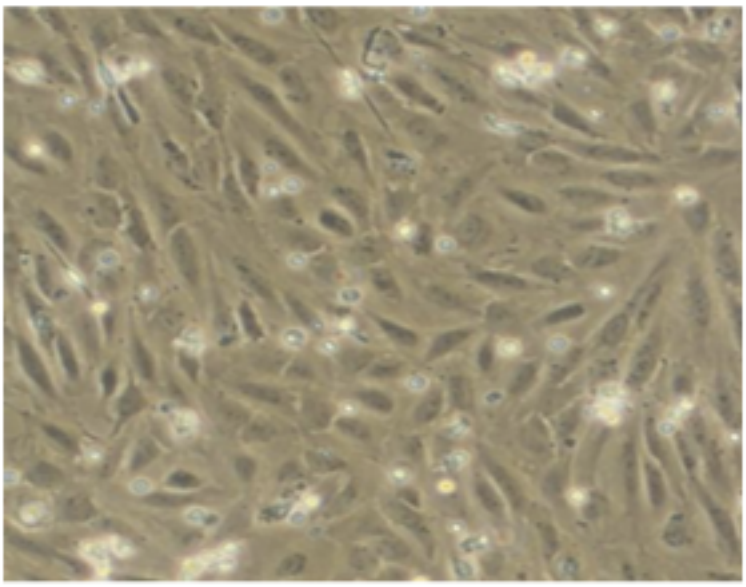

Figure 9. *Phase contrast micrographs of human umbilical vein endothelial cells (HUVEC)*

4.2. Treatment of Cells

For cell splitting, medium M199 was aspirated and cells were shortly washed with phosphate-buffered saline (PBS) without Ca and Mg. Cells were then incubated for 10 minutes with trypsin at 37°C and resuspended in appropriate medium. Cells were centrifuged for 5 minutes at 1500 rpm. The supernatant was removed, and the cell pellet was resuspended. Resuspended cells were transferred in 24 well cell-culture plates and starved in M199 containing 0.1% bovine serum albumin (BSA; Sigma) for 24 hours (h). Before experiments medium was replaced with fresh M199 containing 1.25% FCS. Additionally, HUVEC were treated with drugs ranolazine (125mg/ml), nicorandil (52,5mg/ml) and trimetazidine (84,5 mg/ml) and incubated for 30 minutes. The cells were then treated with or without IL-1β (10ng/ml; R&D Systems) and incubated for 4 h. Dimethyl sulfoxide (DMSO) and 70% ethanol (EtOH) were used as a control. DMSO was also used as solvent for drugs ranolazine and nicorandil. EtOH was used to dissolve trimetazidine.

4.3. Determination of mRNA and complementary DNA (cDNA) preparation

After treatment of cells, culture supernatans were removed, cells were lysed, and mRNA was isolated using High Pure RNA Isolation Kit (Roshe, Basel, Switzerland) according to the manufacturer's instructions. Cells were stimulated as described above, supernatant was removed, and mRNA was isolated with the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. To determine the concentration of RNA, 1µl of each RNA sample was examined on the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). For obtaining cDNA, reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche).

4.4. Real-Time polymerase chain reaction

Specific mRNA levels for target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by real time polymerase chain reaction (RealTime PCR) using LightCycler-RNA Master SYBR Green I (Roche, Basel, Switzerland) according to the manufacturer's instructions.
The general principle (Figure 2): cDNA is denaturated, annealed with primers and a desired fragment within the gene of interest is amplified. In addition, real-time PCR exactly quantifies the amount of PCR product in real time, simultaneously to the amplification. This is possible by addition of a short nucleotide probe labelled with a fluorescent reporter as well as a quencher. The 3`-end of the probe is phosphorylated, so that it cannot be extended during PCR. When the probe is intact the quencher molecule is close enough to the report to suppress the reporter fluorescent signal. As the primers bind and are extended 5`-nuclease activity of the Taq polymerase cleaves the hydrolysis probe and reporter and quencher are released separately. In the cleaved probe the dye is able to emit fluorescence, correlating to the amount of PCR product. Consequently, the total amount of PCR product can be measured

at every time point during the process. Upon comparison with housekeeping gene e.g. human GAPDH the exact amount of the gene of interest expressed in the cell population can be determined.

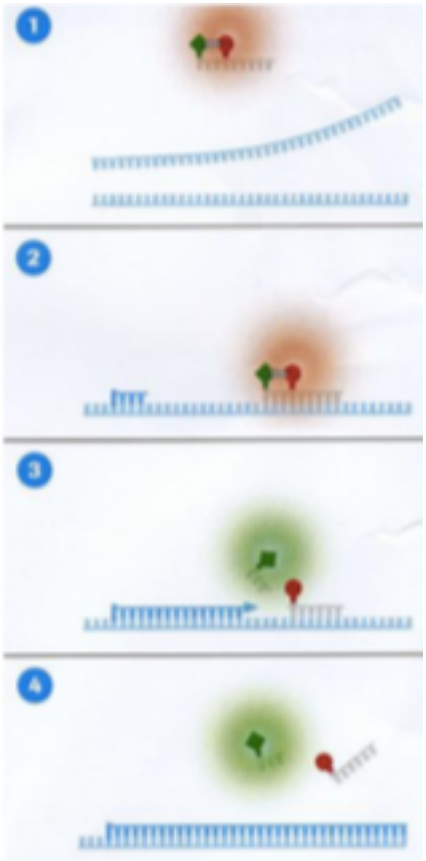

Figure 10. *Mechanism of hydrolysis probe assay*

Specific mRNA levels for ICAM-1, VCAM-1, TF, E-selectin, IL-6, IL-8 were determined by real time PCR. RealTime PCR was performed using LightCyclerTaqMan Master (Roche) according to manufacturer's instruction (Table 1). Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com): GAPDH (forward primer: 5'-agccacatcgctcagacac-3', reverse primer: 5'-gcccaat-acgaccaaatcc-3', UPLprobe #60; Amplicon Size [bp] 66) – VCAM-1 (forward primer: 5'-tgaatctaggaaattggaaaaagg-3', reverse primer: 5'- tgaatctctggatccttaggaaa –3', UPLprobe #39; Amplicon Size [bp] 69) – ICAM-1 (forward primer: 5'-ccttcctcaccgtgtactgg-3', reverse primer: 5'-agcgtagggtaaggttcttgc-3', UPLprobe #71; Amplicon Size [bp] 90) – E-selectin (forward primer: 5' accagcccaggttgaatg-3', reverse primer: 5'- ggttggacaaggctgtgc-3', UPLprobe #86; Amplicon Size [bp] 89).

The amplification starts with an initial incubation at 95 °C for 10 min, followed by 45 cycles of 95°C for 10 seconds, 63°C for 20 seconds and 72°C for 6 sec and a final cooling to 40°C (Table 2). LightCycler Software Version 3.5. (Roche) was used to perform data analysis.

master mix	volume [µl]
forward primer	0,04
reverse primer	0,04
Universal ProbeLibrary probe	0,2
LightCycler 480 Probes Master	10
dH2O	9

Table 2. *Real-time PCR master mix*

Cycles	temperature °C	time
1	95	10min
45	95	10sec
	63	20sec
	72	6sec
1	40	10sec

Table 3. *Thermal cycling program for RT-PCR*

4.5. Flow cytometry

(FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). After removing the medium adherent cells were incubated with a detachment buffer (Dulbecco's PBS without Ca and Mg, PAA, Pashing, Austria) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Boehringer Mannheim GmnH, Mannheim, Germany) and 10 mM ethylenediaminetetraacetic acid (EDTA) (Pierce, Rockford, IL, USA) for 10 minutes. Afterwards, cells were gently removed from the wells and incubated at 4°C at dark with primary antibodies against ICAM-1 (FITC, mouse anti-human CD54, Beckman Coulter, Brea, CA, USA), VCAM-1 (PE-Cy5^{TM} mouse anti-human CD106, BD Pharmingen, San Jose, CA, USA) and E-selectin (PE mouse anti-human CD62E; BD Pharmingen) that were

diluted 1:40 in antibody diluents solution (DAKO North America Inc., Carpinteria, CA, USA) before use. Cells were then washed with 1x PBS, resuspended in fixative solution (FACS Flow, aqua destillata and BD CellfixTM) and mean fluorescence intensity (MFI) was analysed by FACS Diva software (BD)[118].

4.6. Isolation of human polymorphonuclear neutrophils (PMN)

For the isolation of human PMN heparinised (100 U/ml) peripheral venous blood was taken from donors using the vacuette blood collection set (21G needle; Greiner bio-one, Krems-münster, Austria) without tourniquet. For the positive isolation of PMN from whole blood, the Easy-Sep® Human Whole Blood CD66b Positive Selection Kit (Stemcell technologies, Grenoble, France) was used according to manufacturer's instructions. After lysis of erythrocytes, positive isolation was performed by adding carcinoembryonic antigen-related cell adhesion molecule 8 (CD66b) antibodies conjugated to magnetic beads. The vial containing the cells with conjugated magnetic beads was placed into a magnetic field, the fluidic content was discarded, and the remaining cells were resuspended with fresh M199 medium[118].

4.7. Adhesion assay under static conditions

In this experiment we determined the PMN adhesion to HUVEC under static conditions. Medium was removed and the confluent HUVEC monolayer was incubated with 1 ml/well medium alone or medium containing ranolazine (500 μM), nicorandil (500 μM), or trimetazidine (500 μM) for 30 min and subsequently with IL-1β (200 U/ml). After 4 h of incubation, cells were washed three times with medium and subsequently, 1 ml medium containing 1×10^6 PMN was added to the wells and incubated at 37 °C in a 5 % CO_2 atmosphere for the time periods indicated. Afterwards, cells were washed with PBS and cell adhesion was examined under a Zeiss Axiovert 40 CFL light microscope with 10× lens. A Zeiss Axiocam ICc3 camera was used to acquire images. The area covered by granulocytes was measured using the Image-J software (National In- stitute of Health, Bethesda, MD, USA)[118].

4.8. Determination of cell viability

In order to exclude possible cytotoxic effects of the tested substances, lactate dehydrogenase (LDH) leakage was measured in cultures treated as described above using a commercially available assay of photometric determination of LDH activity (Sigma).

4.9. Statistical analysis

All data are presented as mean ± SD. Data were compared by ANOVA followed by Bonferroni correction. Values of $p<0.05$ (2-tailed) were considered as statistically significant.

5. Results

5.1. Effects of ranolazine, nicorandil and trimetazidine on IL-1ß induced mRNA expression of adhesion molecules in HUVEC

HUVEC were cultured as described in the methods section. Cells were incubated with or without IL-1ß (200 U/mL) for 4 h. mRNA was isolated as described in the methods section. Basal expression of ICAM-1, VCAM-1, E-selectin and TF was low. Treatment of cells with IL-1ß increased the expression of adhesion molecules.

Ranolazine (500 µM) alone showed no effect on basal expression of VCAM-1, ICAM-1, TF and E-selectin. Treatment with ranolazine significantly reduced IL-1ß induced expression of ICAM-1 (Figure 11A), VCAM-1 (Figure 11B), E-selectin (Figure 11C) and TF (Figure 11D).

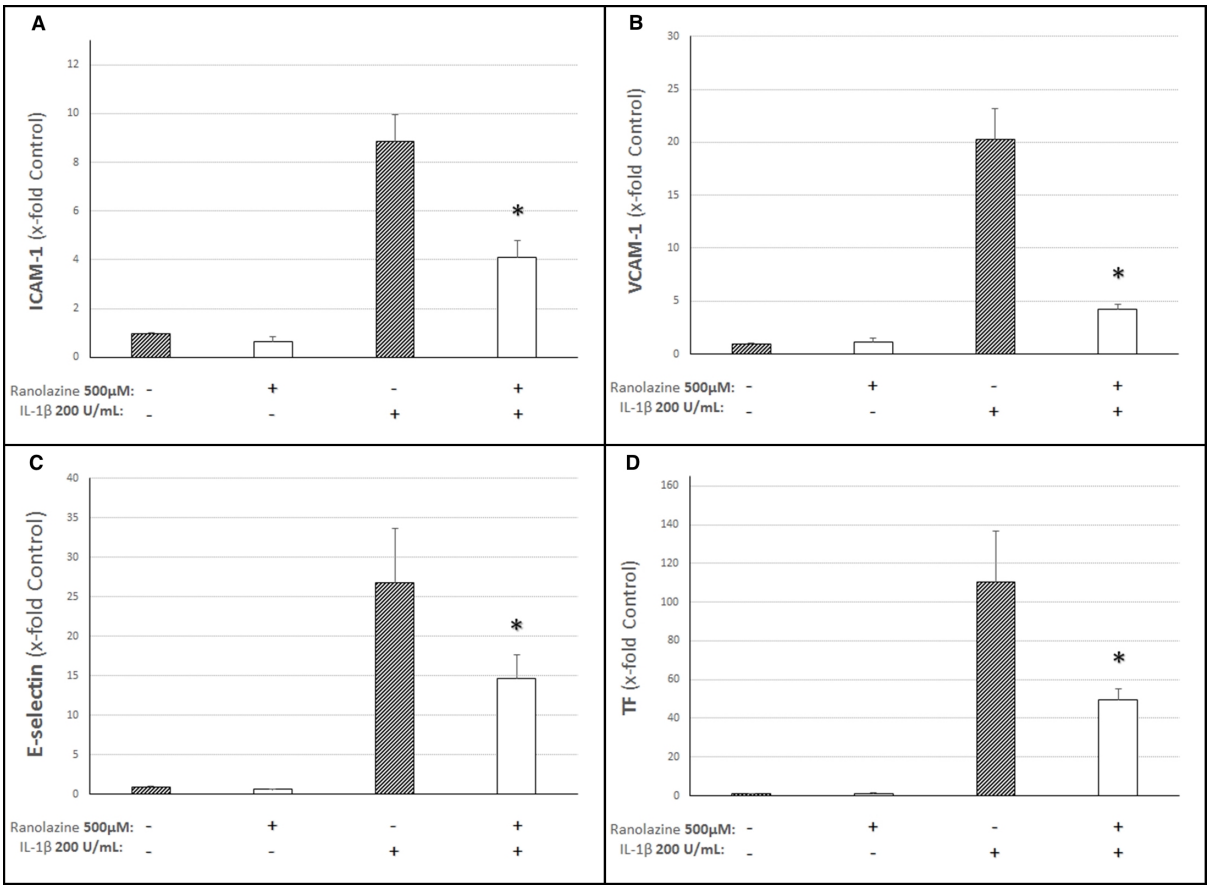

Figure 11. *Effects of ranolazine on IL-1ß induced expression of VCAM-1, ICAM-1, TF and E-selectin. Cells were treated with or without ranolazine (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. mRNA was isolated and RealTime PCR with primers specific for ICAM-1(A), VCAM-1 (B), E-selectin (C), TF(D) and GAPDH was performed as described in Materials and Methods. Data is presented as ration of targed gene/GAPDH in x-fold of controls. *p<0.05 with vs. without ranolazine.*

While ranolazine significantly downregulated IL-1ß induced expression of adhesions molecules, nicorandil (500µM) had no inhibitory effect on IL-1ß induced expression of ICAM-1 (Figure 12A), VCAM-1 (Figure 12B), E-selectin (Figure 12C) and TF (Figure 12D). Nicorandil alone also showed no effect on basal expression of the beforehand mentioned adhesion molecules.

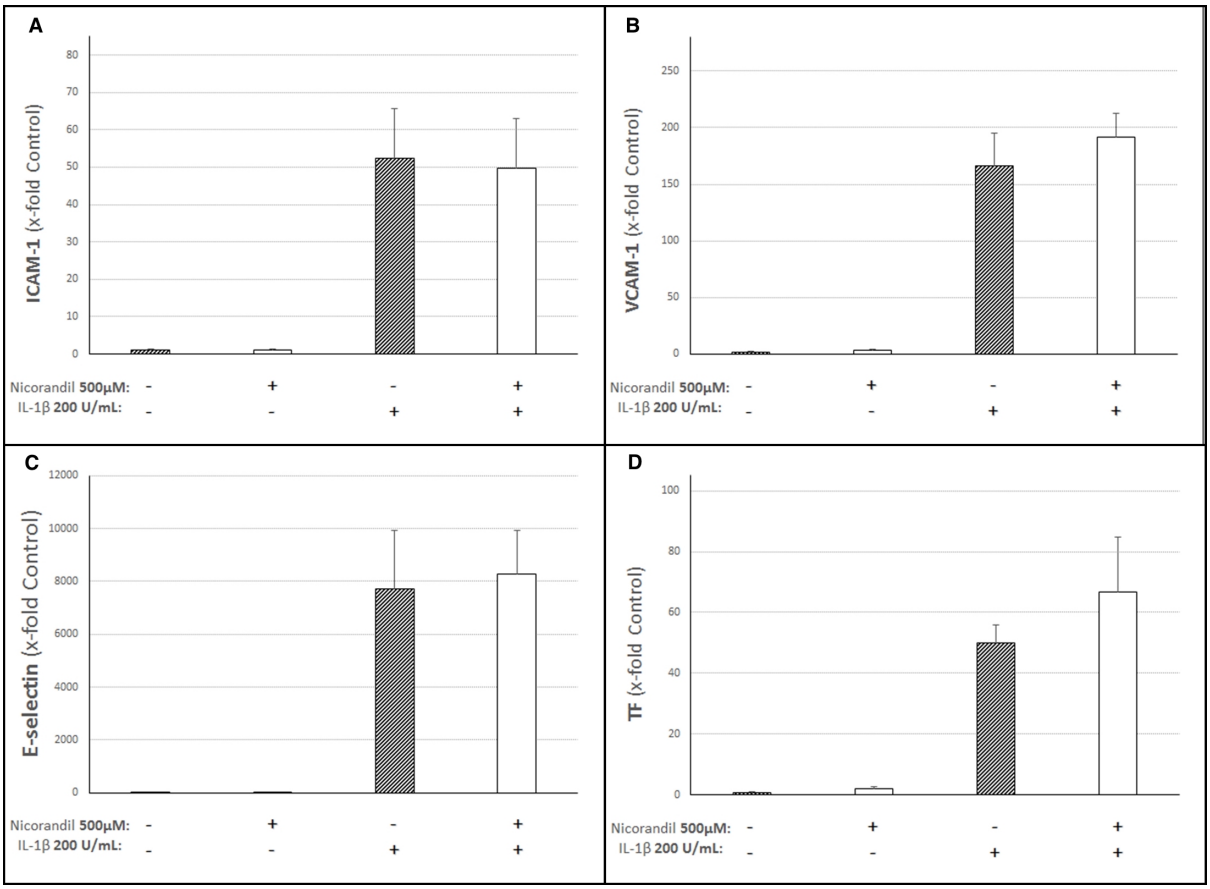

Figure 12. *Effects of nicorandil on IL-1ß induced expression of VCAM-1, ICAM-1, TF and E-selectin. Cells were treated with or without nicorandil (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. mRNA was prepared and RealTime PCR with primers specific for ICAM-1(A), VCAM-1 (B), E-selectin (C), TF(D) and GAPDH was performed as described in Materials and Methods. Data is presented as ration of targed gene/GAPDH in x-fold of controls. *p<0.05 with vs. without nicorandil*

Similar to ranolazine and nicorandil, trimetazidine alone had no effect on basal expression of VCAM-1, ICAM-1, TF and E-selectin. However, treatment with trimetazindine significantly increased IL-1ß induced expression of ICAM-1 (Figure 13A), E-selectin (Figure 13C) and TF (Figure 13D) and significantly reduced IL-1ß induced expression of VCAM-1 (Figure 13B).

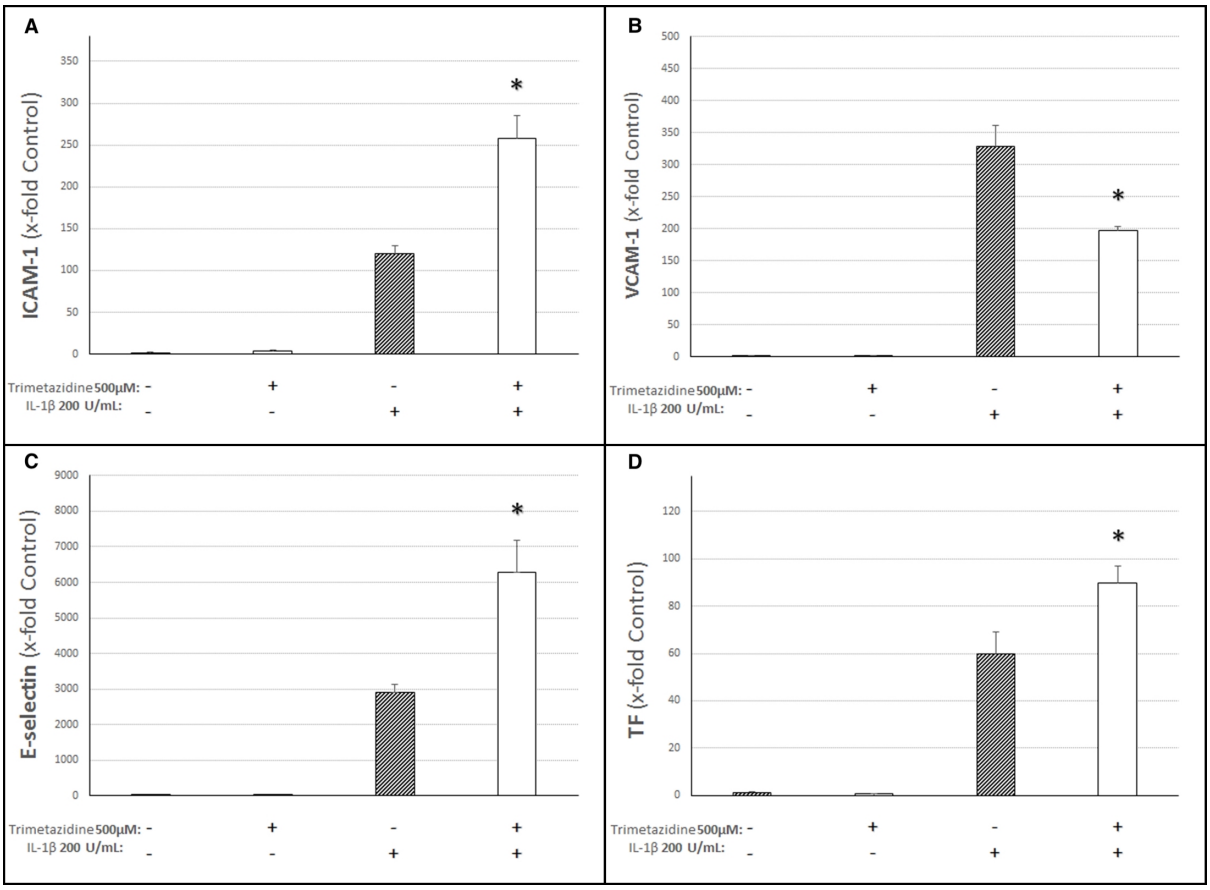

Figure 13. *Effects of trimetazidine on IL-1ß induced expression of VCAM-1, ICAM-1, TF and E-selectin. Cells were treated with or without trimetazidine (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. mRNA was prepared and RealTime PCR with primers specific for ICAM-1(A), VCAM-1 (B), E-selectin (C), TF(D) and GAPDH was performed as described in Materials and Methods. Data is presented as ration of targed gene/GAPDH in x-fold of controls. *p<0.05 with vs. without trimetazidine.*

5.2. Effects of ranolazine, nicorandil and trimetazidine on IL-1ß induced surface expression of adhesions molecules in HUVEC

HUVEC were cultured as described in the methods section. Cells were incubated with or without IL-1ß (200 U/mL) for 4 h. Flow cytometry was performed as described in the methods section. After treatment with IL-1ß for 4 h, the basal expression of adhesion molecules increased.

The IL-1ß induced surface expression of ICAM-1, VCAM-1, E-selectin and TF was significantly downregulated when cells were treated with ranolazine (500µM) (Figure 14).

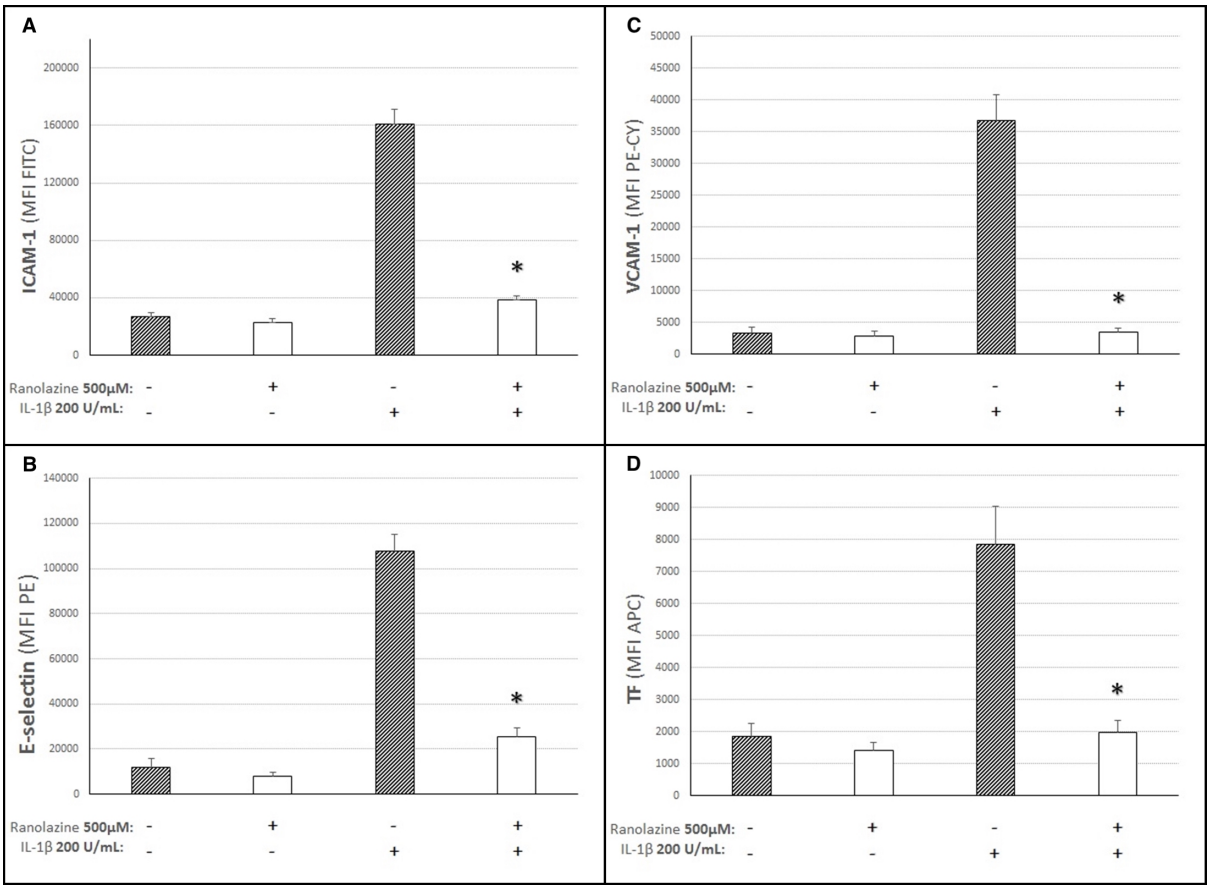

Figure 14. *Effects of ranolazine on IL-1ß induced surface expression of ICAM-1, VCAM-1, E-selectin and TF in HUVEC. Cells were treated with or without ranolazine (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. Thereafter cells were detached, stained with the fluorescence-labelled monoclonal antibodies against ICAM-1(A), VCAM-1(C), E-selectin (B) and TF (D) and analysed by flow cytometry. Values given in MFI and represent mean values ± SD. *p<0.05 for IL-1ß without vs. IL-1ß with ranolazine*.

Nicorandil (500µM) did not reduce IL-1ß induced surface expression of adhesion molecules (Figure 15).

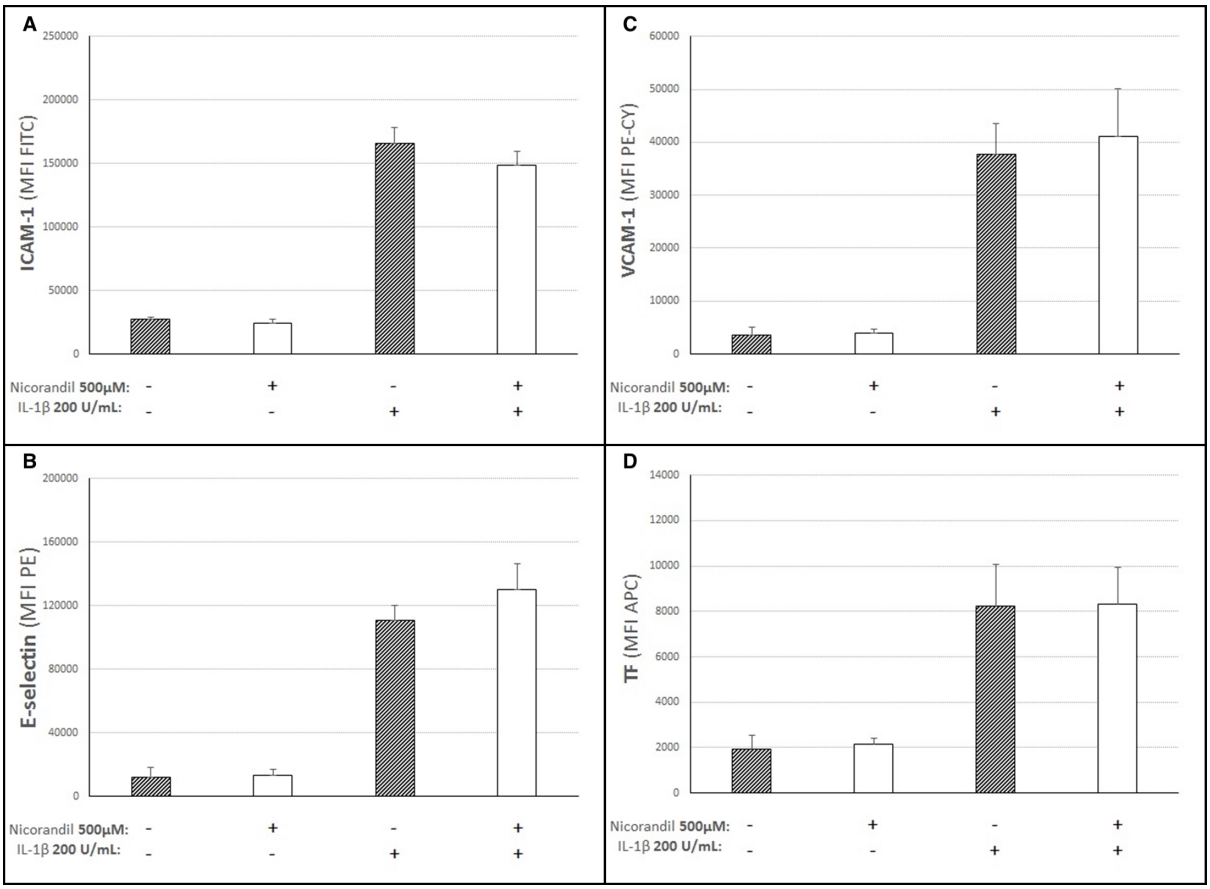

Figure 15. *Effects of nicorandil on IL-1ß induced surface expression of ICAM-1, VCAM-1, E-selectin and TF in HUVEC. Cells were treated with or without nicorandil (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. Thereafter cells were detached, stained with the fluorescence-labelled monoclonal antibodies against ICAM-1(A), VCAM-1(C), E-selectin (B) and TF (D) and analysed by flow cytometry. Values given in MFI and represent mean values ± SD.*

Ranolazine and nicorandil results from this experiment confirmed previously results from RealTime PCR experiment.

Additionally, cells were co-incubated with trimetazidine. Similar to nicorandil it showed no reduction of IL-1ß induced surface expression of ICAM-1, E-selectin and TF. However, the IL-1ß induced surface expression of VCAM-1 was significantly reduced (Figure 16).

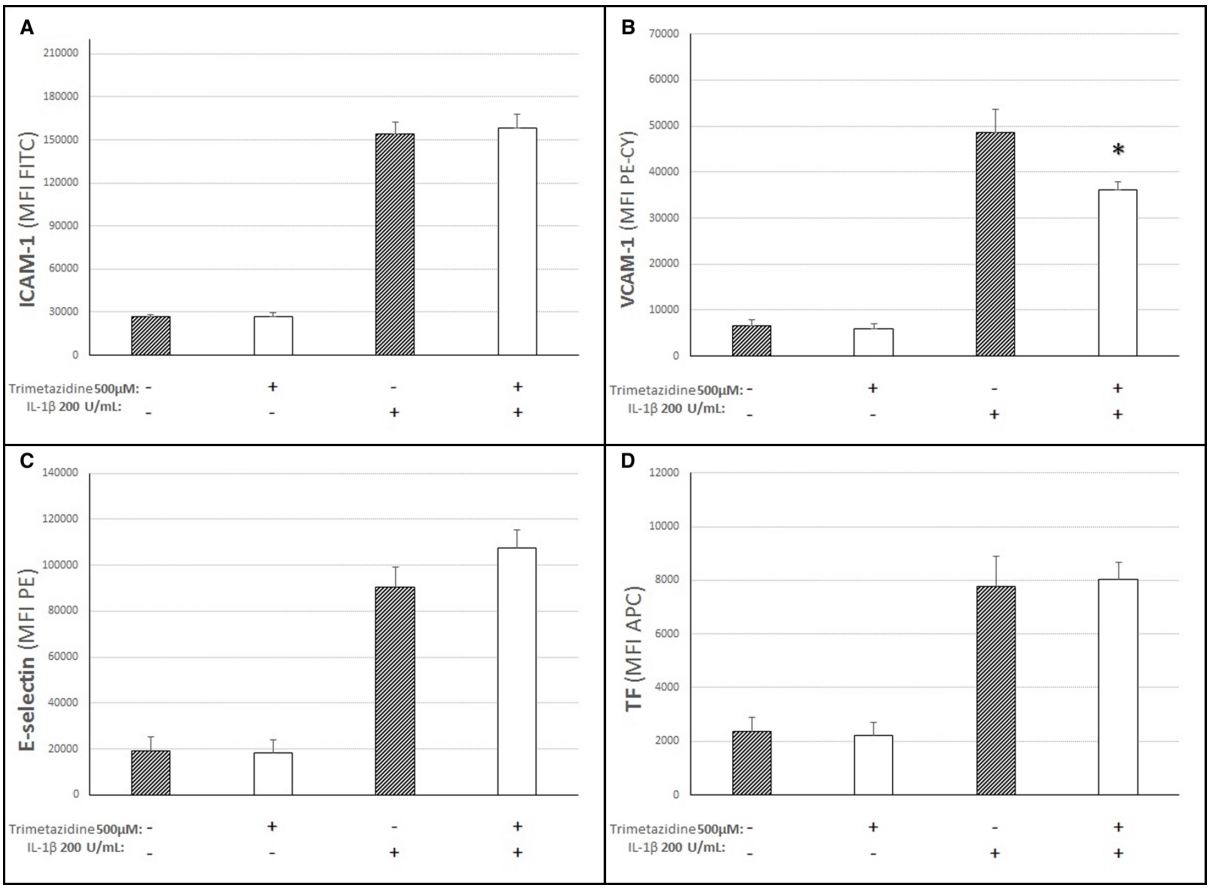

Figure 16. *Effects of trimedazidine on IL-1ß induced surface expression of ICAM-1, VCAM-1, E-selectin and TF in HUVEC. Cells were treated with or without trimetazidine (500µM) alone or together with IL-1ß (200 U/ml) for 4 h. Thereafter cells were detached, stained with the fluorescence-labelled monoclonal antibodies against ICAM-1(A), VCAM-1(B), E-selectin (C) and TF (D) and analysed by flow cytometry. Values given in MFI and represent mean values ± SD. *p<0.05 for IL-1ß without vs. IL-1ß with trimetazidine.*

5.3. Effects of ranolazine, nicorandil and trimetazidine on IL-1ß mediated adhesion of ploymorphonuclear neutrophils to HUVEC

Polymorphonuclear neutrophils (PMN) were collected and treated as described in methods section. HUVEC were incubated with IL-1ß (200 U/ml) for 4 h. Afterwards 1 ml of $1x10^6$ PMN was added and incubated for 5 minutes. IL-1ß induced adhesion of PMN on HUVEC was observed (Figure 17A). This IL-1ß induced PMN adhesion was significantly reduced when cells were pre-incubated for 30 minutes with ranolazine (500µM) (Figure 17B and C).

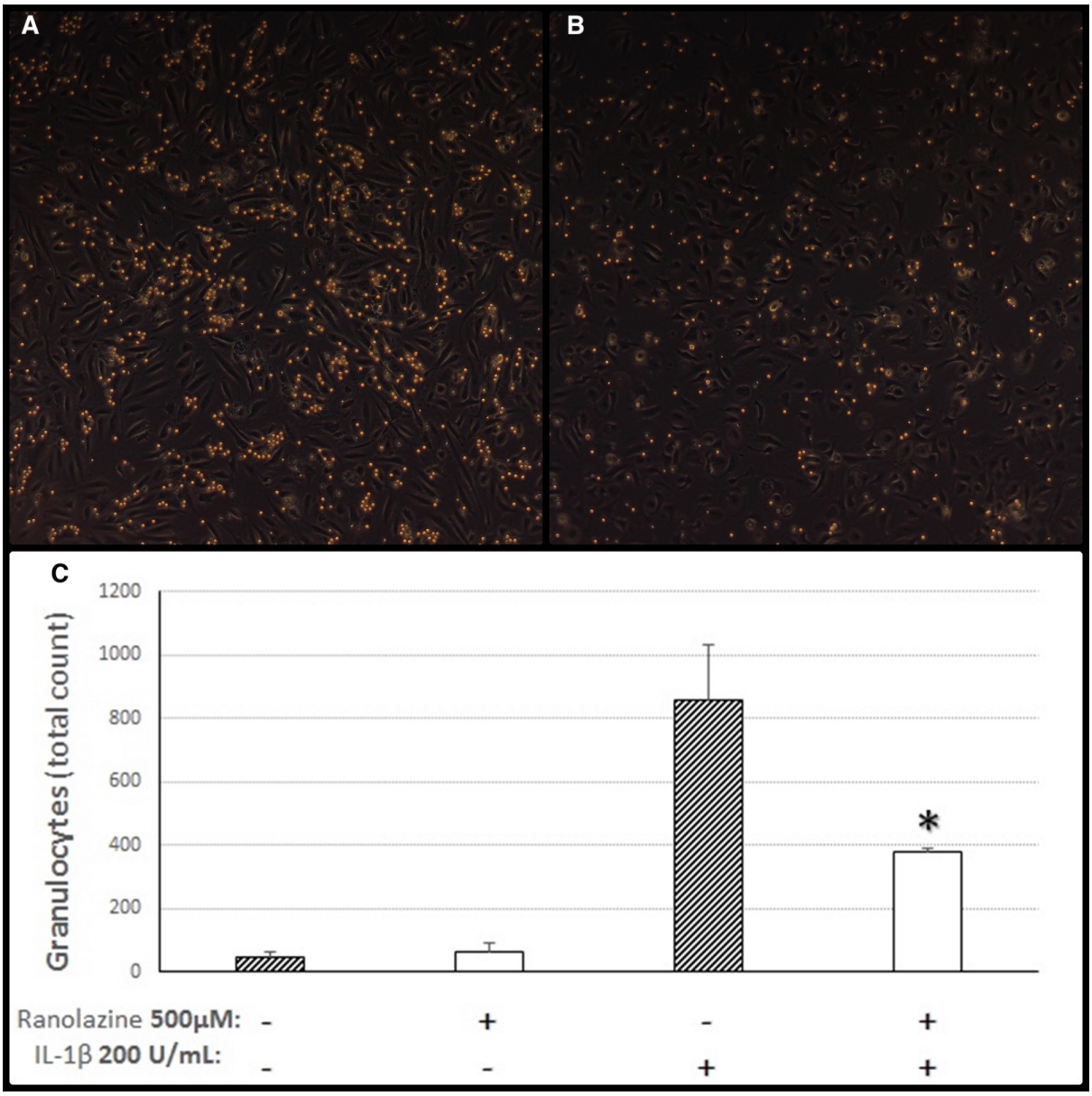

Figure 17. *Effects of ranolazine on IL-1ß induced adhesion of PMN to HUVEC. Adhesion assay under static conditions: HUVEC were treated with IL-1ß for 4 h and incubated with PMN for 5 minutes (A). HUVEC pre-incubated with ranolazine (500µM) for 30 minutes (B). Cell adhesion was examined under a Zeiss Axiovert 40 CFL light microscope with 10x lens. *p<0.05 for IL-1ß+ PMN vs. ranolazine + IL-1ß + PMN*

Addition of nicorandil (500µM) to IL-1ß and PMN treated HUVEC did not result in reduction of IL-1ß mediated granulocytes adhesion (Figure 18).

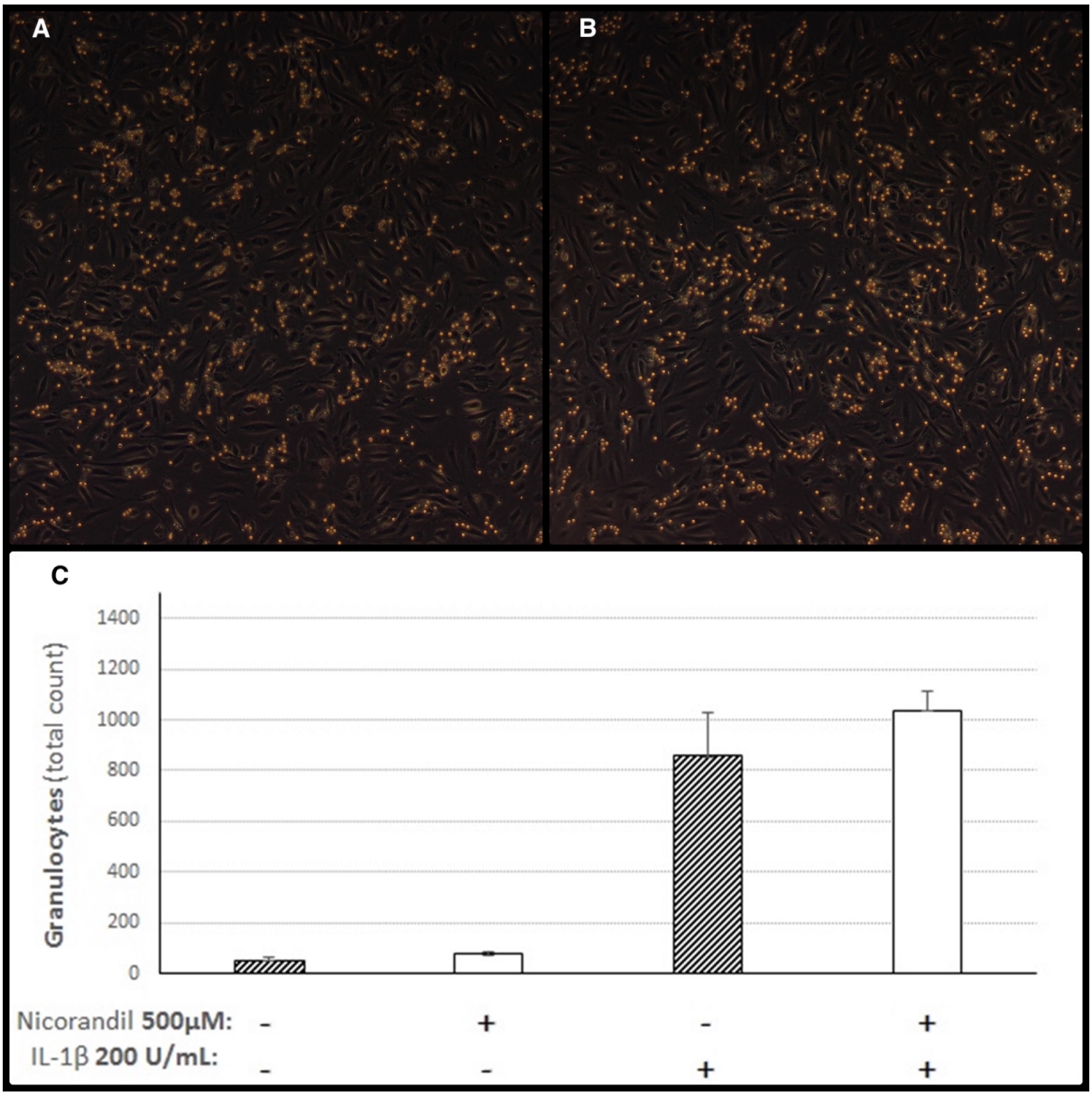

Figure 18. *Effects of nicorandil on IL-1ß induced adhesion of PMN to HUVEC. Adhesion assay under static conditions: HUVEC were treated with IL-1ß for 4 h and incubated with PMN for 5 minutes (A). HUVEC pre-incubated with nicorandil (500µM) for 30 minutes (B). Cell adhesion was examined under a Zeiss Axiovert 40 CFL light microscope with 10x lens. *p<0.05 for IL-1ß+ PMN vs. nicorandil + IL-1ß + PMN*

Similar to nicorandil effects, IL-1ß induced adhesion of PMN was not reduced when HUVEC were pre-incubated with trimetazidine (500µM) (Figure 19).

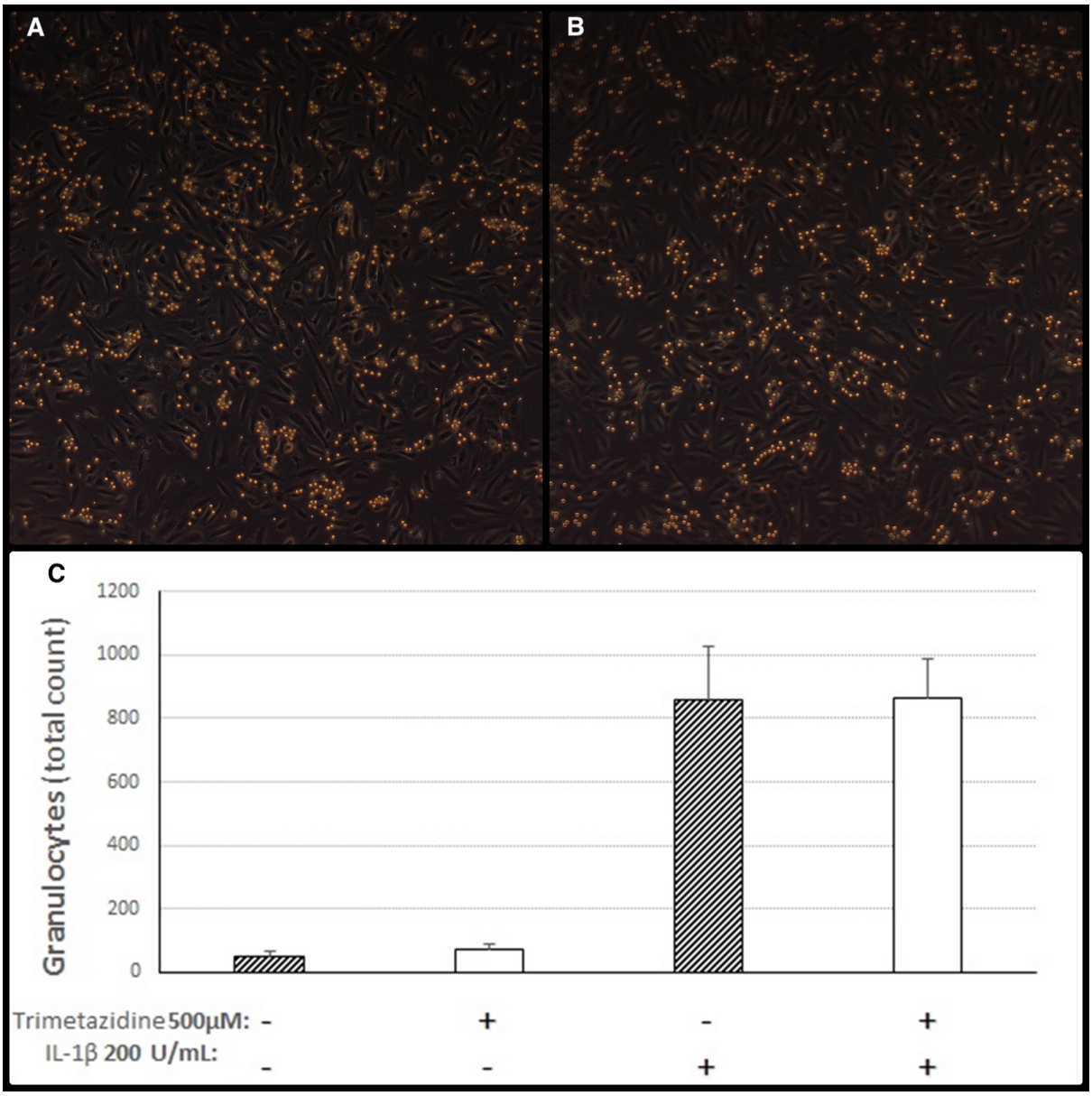

Figure 19. *Effects of trimetazidine on IL-1β induced adhesion of PMN to HUVEC. Adhesion assay under static conditions: HUVEC were treated with IL-1β for 4 h and incubated with PMN for 5 minutes (A). HUVEC pre-incubated with trimetazidine (500µM) for 30 minutes (B). Cell adhesion was examined under a Zeiss Axiovert 40 CFL light microscope with 10x lens. *p<0.05 for IL-1β+ PMN vs. ranolazine + IL-1β + PMN*

5.4. Determination of cell viability

An LDH based toxicity assay excluded any possibly toxic effects of the drugs ranolazine, nicorandil and trimetazidine. No difference has been shown between treated and untreated HUVEC (Figure 20).

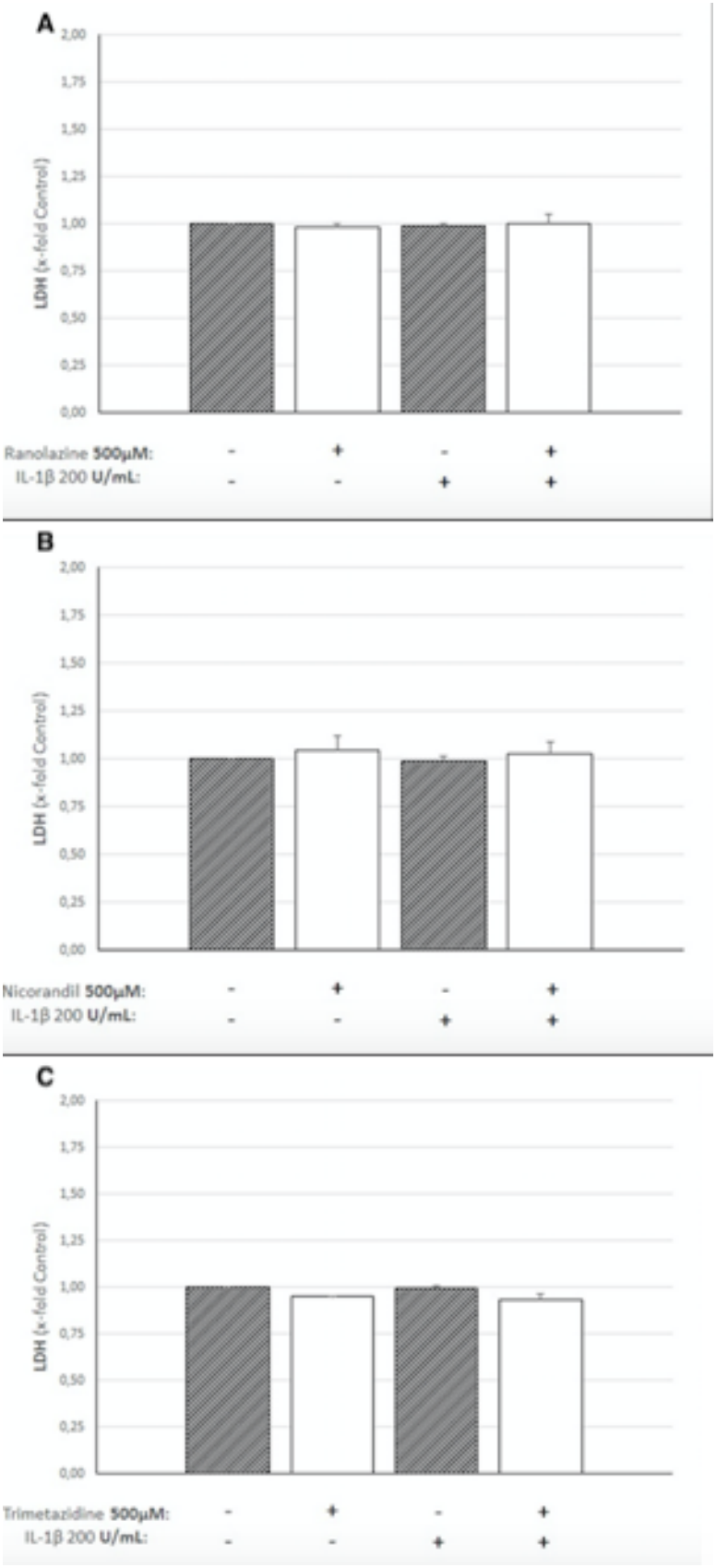

Figure 20. *LDH based toxicity assay. HUVEC were incubated with with IL-1ß and ranolazine (A), nicorandil (B) or trimetazidine (C).*

6. Discussion

In this study we examined the influence of the anti-anginal drugs ranolazine, nicorandil and trimetazidine on the inflammatory process during atherogenesis and their possible anti-inflammatory effect.

Nowadays, the leading cause of death in modern population remain cardiovascular diseases (CVDs). Although CVD refers to any disease of cardiovascular system it is mostly associated with atherosclerosis. Atherosclerosis is today considered to be an inflammatory disease of the vessel wall [45]. Accumulation and peroxidation of LDL-particles within the vessel wall trigger a strong inflammatory response, causing expression of adhesion molecules such as ICAM-1, VCAM-1, E-selectin and TF, and production of cytokines and chemokines that lead to macrophage and T-cell accumulation within the vessel wall. Inflammation within coronary plaques and activation of the coagulation system causes plaque rupture and thrombus formation which can lead to acute coronary events, unstable angina pectoris and sudden cardiac death [49]. Atherosclerosis is also responsible for narrowing of coronary arteries, therefore it represents the most common pathological cause of angina pectoris.
Angina pectoris is a common symptom of IHD and it represents a disproportion between oxygen demand and oxygen consumption [5-8]. Due to its clinical symptoms like pain in the chest, jaw, shoulder, back, or arms, angina limits the normal day activities and reduces the quality of life.

The consequence of ischaemia are reduced oxygen consumption and reduction of ATP formation in the mitochondria. Reduction of ATP synthesis stimulates non-oxidative 'anaerobic' glycolysis and myocardial lactat production leading to cell acidosis [25-29]. When pH is low, energy released for ATP hydrolysis is being used for Ca^{2+} regulation in cytosol and less is available for contractile work [30]. Also, ischaemia leads to tissue hypoxia and cell necrosis. A cytokine cascade is being initiated, in the first line by IL-1ß, causing activation of the microvascular endothelium. Once activated, endothelial cells express selectines, such as E-selectin, that results in attachment and rolling of leukocytes. Adhesion molecules like ICAM-1 and VCAM-1 are also being expressed, causing so adhesion and migration of PMN leading so to further tissue damage [31].

Next to the optimal medical therapy of angina pectoris which includes a combination of 2 and, sometimes 3 different types of anti-anginal therapy (beta-blockers, calcium chanel blockers, statins, ACE inhibitors) the metabolic approach to treating IHD is taking its lead. Drugs such as ranolazine, nicorandil and trimetazidine are modulating the myocardial metabolism and reducing the ischaemic damage.

Ranolazine is a piperazine derivate used in patients that did not have an adequate response with other anti-anginal drugs. As already mentioned, ranolazine has an impact on myocardial metabolism. Ranolazine shifts ATP production away from fatty acid oxidation in favor of more oxygen-efficient carbohydrate oxidation [64]. In several trials it has been shown that

ranolazine monotherapy as well as combination with other anti-anginal drugs leads to prolongation of exercise duration and provides relief to symptomatic patients [66,72]. Ranolazine shows no effect on heart rate or blood pressure.
In this study we presented evidence that ranolazine exhibits anti-inflammatory effects on HUVEC *in vitro*. Furthermore, we could prove that ranolazine significantly reduces the expression of ICAM-1 and VCAM-1, both responsible for the adhesion of monocytes and T-lymphocytes and their preferential binding to the arteria surface [49]. The expression of TF and E-selectine, was also significantly reduced on both mRNA and protein levels. E-selectine is meditating the first step in leukocytes extravasation [50] and plays an important role in inflammatory reaction and development of vulnerable plaque. Treatment with ranolazine reduced granulocyte adhesion to HUVEC. Granulocyte adhesion to the vessel wall and subsequent extravasation into myocardial tissue represents a detrimental step in reperfusion injury [120].

Nicorandil is an anti-anginal drug that has dual mechanism of action. It is an K+ ATP channel agonist and because of its nitrat-like properties (nicorandil has a terminal NO_2 group) it is responsible for vasodilatation, increasing the blood supply to the ischaemic region of the myocardium. Nicorandil, as well as ranolazine, prolonges the duration of exercise and delays the onset of ischaemic ST depression [71,74].
In our study nicorandil did not change IL-1ß induced expression of adhesions molecules on mRNA or protein level. It also did not reduce the granulocyte adhesion.

Trimetazidine was the last of the three drugs we examined. Trimetazidine displays anti-ischaemic effects without inducing any significant haemodynamic changes [103] and like ranolazine and nicorandil it displays its action at the cellular level by improving myocardial energy metabolism [106-110]. The drug acts via partial inhibition of myocardial fatty acid oxidation, specifically, it inhibits the long-chain activity of the enzyme acetyl-CoA C-acyltransferase. Additionally, trimetazidine shows antioxidant effects, limits membrane damage induced by reactive oxygen species and protects tissue from free radicals [110].
We have been able to prove that trimetazdine significantly increased the IL-1ß induced expression of ICAM-1, E-selectine and TF and at the same time the expression of VCAM-1 was significantly reduced on mRNA level. Why does trimetazidine induce the expression of ICAM-1, E-selectin, TF and attenuates the expression of VCAM-1? Future research will be needed to answer this question. In flow cytometry experiment trimetazidine, again, significantly attenuated the IL-1ß induced surface expression of VCAM-1, however it had no effect on IL-1ß induced surface expression of ICAM-1, E-selectin and TF. The IL-1ß induced granulocyte adhesion was also not affected by trimetazidine.

7. Conclusion

In this study we present evidence that ranolazine suppresses the IL-1ß induced expression of adhesion molecules ICAM-1, VCAM-1, E-selectin and TF and therefore it has an anti-inflammatory effect. If these anti-inflammatory effects are also effective *in vivo,* administration of ranolazine in patients with angina pectoris would not only improve the clinical symptoms of angina pectoris and lead to prolongation of exercise duration, but it would also prevent macrophage and T-cell accumulation within the vessel wall and prevent further amplification of inflammatory reaction caused by these cells. Altogether, by doing so ranolazine could also prevent the plaque rupture and thrombus formation which can lead to acute coronary events and even to sudden cardiac death.

Even though trimetazidine significantly suppressed only the expression of VCAM-1, its effects and mechanisms of action should be further examined.

Nicorandil seems not to have any anti-inflammatory effects in HUVEC.

8. References

1. Wolff, A.A., et al., *Metabolic approaches to the treatment of ischemic heart disease: the clinicians' perspective.* Heart Fail Rev, 2002. **7**(2): p. 187-203.
2. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-16.
3. Stanley, W.C. and M. Marzilli, *Metabolic therapy in the treatment of ischaemic heart disease: the pharmacology of trimetazidine.* Fundam Clin Pharmacol, 2003. **17**(2): p. 133-45.
4. Jerling, M., et al., *Studies to investigate the pharmacokinetic interactions between ranolazine and ketoconazole, diltiazem, or simvastatin during combined administration in healthy subjects.* J Clin Pharmacol, 2005. **45**(4): p. 422-33.
5. Wong, N.D., *Epidemiological studies of CHD and the evolution of preventive cardiology.* Nat Rev Cardiol, 2014. **11**(5): p. 276-89.
6. Kannel, W.B. and M. Feinleib, *Natural history of angina pectoris in the Framingham study. Prognosis and survival.* Am J Cardiol, 1972. **29**(2): p. 154-63.
7. *WHO publishes definitive atlas on global heart disease and stroke epidemic.* Indian J Med Sci, 2004. **58**(9): p. 405-6.
8. Blomer, H., *[Physiopathology of angina pectoris].* Wien Med Wochenschr, 1984. **134**(23-24): p. 527-34.
9. Gibbons, R.J., et al., *ACC/AHA 2002 guideline update for the management of patients with chronic stable angina--summary article: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines (Committee on the Management of Patients With Chronic Stable Angina).* J Am Coll Cardiol, 2003. **41**(1): p. 159-68.
10. Fox, K., et al., *Guidelines on the management of stable angina pectoris: executive summary: The Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology.* Eur Heart J, 2006. **27**(11): p. 1341-81.
11. *Five-year clinical and functional outcome comparing bypass surgery and angioplasty in patients with multivessel coronary disease. A multicenter randomized trial. Writing Group for the Bypass Angioplasty Revascularization Investigation (BARI) Investigators.* JAMA, 1997. **277**(9): p. 715-21.
12. Holubkov, R., et al., *Angina 1 year after percutaneous coronary intervention: a report from the NHLBI Dynamic Registry.* Am Heart J, 2002. **144**(5): p. 826-33.
13. Serruys, P.W., et al., *Comparison of coronary-artery bypass surgery and stenting for the treatment of multivessel disease.* N Engl J Med, 2001. **344**(15): p. 1117-24.
14. Parker, J.O., *Angina pectoris: a review of current and emerging therapies.* Am J Manag Care, 2004. **10**(11 Suppl): p. S332-8.
15. Valgimigli, M. and S. Biscaglia, *Stable angina pectoris.* Curr Atheroscler Rep, 2014. **16**(7): p. 422.
16. Siama, K., et al., *Stable angina pectoris: current medical treatment.* Curr Pharm Des, 2013. **19**(9): p. 1569-80.

17. Poole-Wilson, P.A., et al., *Effect of long-acting nifedipine on mortality and cardiovascular morbidity in patients with stable angina requiring treatment (ACTION trial): randomised controlled trial.* Lancet, 2004. **364**(9437): p. 849-57.
18. Group, B.D.S., et al., *A randomized trial of therapies for type 2 diabetes and coronary artery disease.* N Engl J Med, 2009. **360**(24): p. 2503-15.
19. Henderson, R.A., et al., *Seven-year outcome in the RITA-2 trial: coronary angioplasty versus medical therapy.* J Am Coll Cardiol, 2003. **42**(7): p. 1161-70.
20. Campeau, L., *Letter: Grading of angina pectoris.* Circulation, 1976. **54**(3): p. 522-3.
21. Maseri, A., et al., *Mechanisms and significance of cardiac ischemic pain.* Prog Cardiovasc Dis, 1992. **35**(1): p. 1-18.
22. Cross, H.R., *Trimetazidine for stable angina pectoris.* Expert Opin Pharmacother, 2001. **2**(5): p. 857-75.
23. McGillion, M., et al., *Recommendations for advancing the care of Canadians living with refractory angina pectoris: a Canadian Cardiovascular Society position statement.* Can J Cardiol, 2009. **25**(7): p. 399-401.
24. Vlodaver, Z., H.N. Neufeld, and J.E. Edwards, *Pathology of angina pectoris.* Circulation, 1972. **46**(6): p. 1048-64.
25. Kolwicz, S.C., Jr., S. Purohit, and R. Tian, *Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes.* Circ Res, 2013. **113**(5): p. 603-16.
26. Lewandowski, E.D., et al., *Mitochondrial preference for short chain fatty acid oxidation during coronary artery constriction.* Circulation, 2002. **105**(3): p. 367-72.
27. Liedtke, A.J., *Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart.* Prog Cardiovasc Dis, 1981. **23**(5): p. 321-36.
28. McNulty, P.H., et al., *Glucose metabolism distal to a critical coronary stenosis in a canine model of low-flow myocardial ischemia.* J Clin Invest, 1996. **98**(1): p. 62-9.
29. Stanley, W.C., et al., *Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions.* Cardiovasc Res, 1997. **33**(2): p. 243-57.
30. Fabiato, A. and F. Fabiato, *Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles.* J Physiol, 1978. **276**: p. 233-55.
31. Frohlich, G.M., et al., *Myocardial reperfusion injury: looking beyond primary PCI.* Eur Heart J, 2013. **34**(23): p. 1714-22.
32. Zaman, M.J., et al., *Presentation of stable angina pectoris among women and South Asian people.* CMAJ, 2008. **179**(7): p. 659-67.
33. Gelfand, M.L., H. Kloth, and L. Goodkin, *The medical treatment of angina pectoris.* Am Fam Physician, 1976. **13**(4): p. 84-90.
34. Zelis, R., et al., *Angina pectoris. Diagnosis and treatment.* Postgrad Med, 1976. **59**(5): p. 179-88.
35. Banasiak, W., et al., *Pharmacotherapy in patients with stable coronary artery disease treated on an outpatient basis in Poland. Results of the multicentre RECENT study.* Kardiol Pol, 2008. **66**(6): p. 642-9; discussion 650.

36. Sodi-Pallares, D., et al., *The polarizing treatment of acute myocardial infarction. Possibility of its use in other cardiovascular conditions.* Dis Chest, 1963. **43**: p. 424-32.
37. Sodi-Pallares, D., et al., *Effects of an intravenous infusion of a potassium-glucose-insulin solution on the electrocardiographic signs of myocardial infarction. A preliminary clinical report.* Am J Cardiol, 1962. **9**: p. 166-81.
38. Fath-Ordoubadi, F. and K.J. Beatt, *Glucose-insulin-potassium therapy for treatment of acute myocardial infarction: an overview of randomized placebo-controlled trials.* Circulation, 1997. **96**(4): p. 1152-6.
39. Rogers, W.J., et al., *Reduction of hospital mortality rate of acute myocardial infarction with glucose-insulin-potassium infusion.* Am Heart J, 1976. **92**(4): p. 441-54.
40. Oliver, M.F. and L.H. Opie, *Effects of glucose and fatty acids on myocardial ischaemia and arrhythmias.* Lancet, 1994. **343**(8890): p. 155-8.
41. Rowe, M.J., et al., *Effect of a nicotinic-acid analogue on raised plasma-free-fatty-acids after acute myocardial infarction.* Lancet, 1973. **2**(7833): p. 814-8.
42. Russell, D.C. and M.F. Oliver, *Effect of antilipolytic therapy on ST segment elevation during myocardial ischaemia in man.* Br Heart J, 1978. **40**(2): p. 117-23.
43. Murray, C.J. and A.D. Lopez, *Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study.* Lancet, 1997. **349**(9063): p. 1436-42.
44. Fan, J. and T. Watanabe, *Inflammatory reactions in the pathogenesis of atherosclerosis.* J Atheroscler Thromb, 2003. **10**(2): p. 63-71.
45. Singh, R.B., et al., *Pathogenesis of atherosclerosis: A multifactorial process.* Exp Clin Cardiol, 2002. **7**(1): p. 40-53.
46. Libby, P., et al., *Inflammation in atherosclerosis: transition from theory to practice.* Circ J, 2010. **74**(2): p. 213-20.
47. Stary, H.C., et al., *A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association.* Arterioscler Thromb, 1992. **12**(1): p. 120-34.
48. Shih, D.M., et al., *Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis.* J Biol Chem, 2000. **275**(23): p. 17527-35.
49. Skalen, K., et al., *Subendothelial retention of atherogenic lipoproteins in early atherosclerosis.* Nature, 2002. **417**(6890): p. 750-4.
50. Watanabe, T. and J. Fan, *Atherosclerosis and inflammation mononuclear cell recruitment and adhesion molecules with reference to the implication of ICAM-1/LFA-1 pathway in atherogenesis.* Int J Cardiol, 1998. **66 Suppl 1**: p. S45-53; discussion S55.
51. Dong, Z.M., et al., *The combined role of P- and E-selectins in atherosclerosis.* J Clin Invest, 1998. **102**(1): p. 145-52.
52. Smith, J.D., et al., *Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E.* Proc Natl Acad Sci U S A, 1995. **92**(18): p. 8264-8.

53. Napoli, C., et al., *Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions.* J Clin Invest, 1997. **100**(11): p. 2680-90.
54. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
55. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.
56. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
57. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
58. Mach, F., et al., *Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor.* Circulation, 1997. **96**(2): p. 396-9.
59. Packard, R.R. and P. Libby, *Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction.* Clin Chem, 2008. **54**(1): p. 24-38.
60. Cocco, G., et al., *Effects of a new metabolic modulator, ranolazine, on exercise tolerance in angina pectoris patients treated with beta-blocker or diltiazem.* J Cardiovasc Pharmacol, 1992. **20**(1): p. 131-8.
61. Jerling, M., *Clinical pharmacokinetics of ranolazine.* Clin Pharmacokinet, 2006. **45**(5): p. 469-91.
62. Chaitman, B.R., et al., *Anti-ischemic effects and long-term survival during ranolazine monotherapy in patients with chronic severe angina.* J Am Coll Cardiol, 2004. **43**(8): p. 1375-82.
63. McCormack, J.G., et al., *Ranolazine stimulates glucose oxidation in normoxic, ischemic, and reperfused ischemic rat hearts.* Circulation, 1996. **93**(1): p. 135-42.
64. Clarke, B., K.M. Wyatt, and J.G. McCormack, *Ranolazine increases active pyruvate dehydrogenase in perfused normoxic rat hearts: evidence for an indirect mechanism.* J Mol Cell Cardiol, 1996. **28**(2): p. 341-50.
65. Hayashida, W., et al., *Effects of ranolazine on left ventricular regional diastolic function in patients with ischemic heart disease.* Cardiovasc Drugs Ther, 1994. **8**(5): p. 741-7.
66. Chaitman, B.R., et al., *Effects of ranolazine with atenolol, amlodipine, or diltiazem on exercise tolerance and angina frequency in patients with severe chronic angina: a randomized controlled trial.* JAMA, 2004. **291**(3): p. 309-16.
67. Antzelevitch, C., et al., *Electrophysiologic properties and antiarrhythmic actions of a novel antianginal agent.* J Cardiovasc Pharmacol Ther, 2004. **9 Suppl 1**: p. S65-83.
68. Abdallah, H. and M. Jerling, *Effect of hepatic impairment on the multiple-dose pharmacokinetics of ranolazine sustained-release tablets.* J Clin Pharmacol, 2005. **45**(7): p. 802-9.
69. Herron, W.J., J. Eadie, and A.D. Penman, *Estimation of ranolazine and eleven phase I metabolites in human plasma by liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry with selected-ion monitoring.* J Chromatogr A, 1995. **712**(1): p. 55-60.

70. Penman, A.D., et al., *The characterization of the metabolites of ranolazine in man by liquid chromatography mass spectrometry.* Rapid Commun Mass Spectrom, 1995. **9**(14): p. 1418-30.
71. Rousseau, M.F., et al., *Comparative efficacy of ranolazine versus atenolol for chronic angina pectoris.* Am J Cardiol, 2005. **95**(3): p. 311-6.
72. Hayata, N., H. Araki, and M. Nakamura, *Effects of nicorandil on exercise tolerance in patients with stable effort angina: a double-blind study.* Am Heart J, 1986. **112**(6): p. 1245-50.
73. Nishimura, M., et al., *Oral nicorandil to reduce cardiac death after coronary revascularization in hemodialysis patients: a randomized trial.* Am J Kidney Dis, 2009. **54**(2): p. 307-17.
74. Sakata, Y., et al., *Oral treatment with nicorandil at discharge is associated with reduced mortality after acute myocardial infarction.* J Cardiol, 2012. **59**(1): p. 14-21.
75. Loubani, M. and M. Galinanes, *Long-term administration of nicorandil abolishes ischemic and pharmacologic preconditioning of the human myocardium: role of mitochondrial adenosine triphosphate-dependent potassium channels.* J Thorac Cardiovasc Surg, 2002. **124**(4): p. 750-7.
76. Gross, G.J., et al., *Enhanced subendocardial perfusion distal to a flow-limiting coronary artery stenosis in dogs: comparative effects of nicorandil, a potential new antianginal agent, and nitroglycerin.* J Cardiovasc Pharmacol, 1985. **7**(5): p. 977-82.
77. Lamping, K.A., et al., *Effects of nicorandil and nifedipine on protection of ischemic myocardium.* J Cardiovasc Pharmacol, 1984. **6**(3): p. 536-42.
78. Thormann, J., et al., *Effectiveness of nicorandil (SG-75), a new long-acting drug with nitroglycerin effects, in patients with coronary artery disease: improved left ventricular function and regional wall motion and abolition of pacing-induced angina.* J Cardiovasc Pharmacol, 1983. **5**(3): p. 371-7.
79. Krumenacker, M. and E. Roland, *Clinical profile of nicorandil: an overview of its hemodynamic properties and therapeutic efficacy.* J Cardiovasc Pharmacol, 1992. **20 Suppl 3**: p. S93-102.
80. Frampton, J., M.M. Buckley, and A. Fitton, *Nicorandil. A review of its pharmacology and therapeutic efficacy in angina pectoris.* Drugs, 1992. **44**(4): p. 625-55.
81. Patel, D.J., H.J. Purcell, and K.M. Fox, *Cardioprotection by opening of the K(ATP) channel in unstable angina. Is this a clinical manifestation of myocardial preconditioning? Results of a randomized study with nicorandil. CESAR 2 investigation. Clinical European studies in angina and revascularization.* Eur Heart J, 1999. **20**(1): p. 51-7.
82. Holzmann, S., et al., *Pharmacological interaction experiments differentiate between glibenclamide-sensitive K+ channels and cyclic GMP as components of vasodilation by nicorandil.* Eur J Pharmacol, 1992. **215**(1): p. 1-7.
83. Cook, N.S., *The pharmacology of potassium channels and their therapeutic potential.* Trends Pharmacol Sci, 1988. **9**(1): p. 21-8.
84. Flaherty, J.T., *Nitrate tolerance. A review of the evidence.* Drugs, 1989. **37**(4): p. 523-50.

85. Holzmann, S., *Cyclic GMP as possible mediator of coronary arterial relaxation by nicorandil (SG-75).* J Cardiovasc Pharmacol, 1983. **5**(3): p. 364-70.
86. Suryapranata, H. and D. MacLeod, *Nicorandil and cardiovascular performance in patients with coronary artery disease.* J Cardiovasc Pharmacol, 1992. **20 Suppl 3**: p. S45-51.
87. Hiraoka, M. and Z. Fan, *Activation of ATP-sensitive outward K+ current by nicorandil (2-nicotinamidoethyl nitrate) in isolated ventricular myocytes.* J Pharmacol Exp Ther, 1989. **250**(1): p. 278-85.
88. Ghosh, S., N.B. Standen, and M. Galinanes, *Preconditioning the human myocardium by simulated ischemia: studies on the early and delayed protection.* Cardiovasc Res, 2000. **45**(2): p. 339-50.
89. Speechly-Dick, M.E., G.J. Grover, and D.M. Yellon, *Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model.* Circ Res, 1995. **77**(5): p. 1030-5.
90. Holmuhamedov, E.L., et al., *Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function.* Am J Physiol, 1998. **275**(5 Pt 2): p. H1567-76.
91. Liu, Y., et al., *Mitochondrial ATP-dependent potassium channels. Viable candidate effectors of ischemic preconditioning.* Ann N Y Acad Sci, 1999. **874**: p. 27-37.
92. Hester, R.K., *Effects of 2-nicotinamidoethyl nitrate on agonist-sensitive Ca++ release and Ca++ entry in rabbit aorta.* J Pharmacol Exp Ther, 1985. **233**(1): p. 100-11.
93. Murakami, K., H. Karaki, and N. Urakawa, *Comparison of the inhibitory effects of nicorandil, nitroglycerin and isosorbide dinitrate on vascular smooth muscle of rabbit aorta.* Eur J Pharmacol, 1987. **141**(2): p. 195-202.
94. Morimoto, S., et al., *Effect of nicorandil on the cytosolic free calcium concentration and microsomal (Ca2+ + Mg2+)-ATPase activity of vascular smooth muscle cells.* J Cardiovasc Pharmacol, 1987. **10 Suppl 8**: p. S31-7.
95. Sakai, K., et al., *Nicorandil metabolism in rat myocardial mitochondria.* J Cardiovasc Pharmacol, 2000. **35**(5): p. 723-8.
96. Frydman, A., *Pharmacokinetic profile of nicorandil in humans: an overview.* J Cardiovasc Pharmacol, 1992. **20 Suppl 3**: p. S34-44.
97. Frydman, A.M., et al., *Pharmacokinetics of nicorandil.* Am J Cardiol, 1989. **63**(21): p. 25J-33J.
98. Reimann, F., F.M. Ashcroft, and F.M. Gribble, *Structural basis for the interference between nicorandil and sulfonylurea action.* Diabetes, 2001. **50**(10): p. 2253-9.
99. Babenko, A.P., G. Gonzalez, and J. Bryan, *Pharmaco-topology of sulfonylurea receptors. Separate domains of the regulatory subunits of K(ATP) channel isoforms are required for selective interaction with K(+) channel openers.* J Biol Chem, 2000. **275**(2): p. 717-20.
100. Ishizuka, N., et al., *Hypotensive interaction of sildenafil and nicorandil in rats through the cGMP pathway but not by K(ATP) channel activation.* Jpn J Pharmacol, 2000. **84**(3): p. 316-24.

101. Jiang, J., et al., *Oral nicorandil reduces ischemic attacks in patients with stable angina: A prospective, multicenter, open-label, randomized, controlled study.* Int J Cardiol, 2016. **224**: p. 183-187.
102. Boddeke, E., et al., *New anti-ischaemic drugs: cytoprotective action with no primary haemodynamic effects.* Trends Pharmacol Sci, 1989. **10**(10): p. 397-400.
103. Dalla-Volta, S., et al., *Comparison of trimetazidine with nifedipine in effort angina: a double-blind, crossover study.* Cardiovasc Drugs Ther, 1990. **4 Suppl 4**: p. 853-9.
104. Sellier, P., et al., *Acute effects of trimetazidine evaluated by exercise testing.* Eur J Clin Pharmacol, 1987. **33**(2): p. 205-7.
105. Aussedat, J., et al., *Improvement of long-term preservation of isolated arrested rat heart: beneficial effect of the antiischemic agent trimetazidine.* J Cardiovasc Pharmacol, 1993. **21**(1): p. 128-35.
106. Boucher, F.R., D.J. Hearse, and L.H. Opie, *Effects of trimetazidine on ischemic contracture in isolated perfused rat hearts.* J Cardiovasc Pharmacol, 1994. **24**(1): p. 45-9.
107. Fantini, E., et al., *Some biochemical aspects of the protective effect of trimetazidine on rat cardiomyocytes during hypoxia and reoxygenation.* J Mol Cell Cardiol, 1994. **26**(8): p. 949-58.
108. McClellan, K.J. and G.L. Plosker, *Trimetazidine. A review of its use in stable angina pectoris and other coronary conditions.* Drugs, 1999. **58**(1): p. 143-57.
109. Stanley, W.C., *Partial fatty acid oxidation inhibitors for stable angina.* Expert Opin Investig Drugs, 2002. **11**(5): p. 615-29.
110. Kantor, P.F., et al., *The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase.* Circ Res, 2000. **86**(5): p. 580-8.
111. Lopaschuk, G.D., *Optimizing cardiac energy metabolism: how can fatty acid and carbohydrate metabolism be manipulated?* Coron Artery Dis, 2001. **12 Suppl 1**: p. S8-11.
112. Iskesen, I., et al., *Trimetazidine reduces oxidative stress in cardiac surgery.* Circ J, 2006. **70**(9): p. 1169-73.
113. Dezsi, C.A., *Trimetazidine in Practice: Review of the Clinical and Experimental Evidence.* Am J Ther, 2016. **23**(3): p. e871-9.
114. Marti Masso, J.F., et al., *Trimetazidine induces parkinsonism, gait disorders and tremor.* Therapie, 2005. **60**(4): p. 419-22.
115. Jackson, P.J., et al., *Identification of trimetazidine metabolites in human urine and plasma.* Xenobiotica, 1996. **26**(2): p. 221-8.
116. Szwed, H., et al., *Combination treatment in stable effort angina using trimetazidine and metoprolol: results of a randomized, double-blind, multicentre study (TRIMPOL II). TRIMetazidine in POLand.* Eur Heart J, 2001. **22**(24): p. 2267-74.
117. Belardinelli, R., *Trimetazidine and the contractile response of dysfunctional myocardium in ischaemic cardiomyopathy.* Rev Port Cardiol, 2000. **19 Suppl 5**: p. V35-9.
118. Krychtiuk, K.A., et al., *Levosimendan exerts anti-inflammatory effects on cardiac myocytes and endothelial cells in vitro.* Thromb Haemost, 2015. **113**(2): p. 350-62.

9. Appendix

9.1. Abbrevations

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndromes
AHA	American Heart Association
ATP	Adenosine triphosphate
AUC	Area under the curve
BSA	Bovine serum albumin
CAD	Coronary artery disease
cGMP	Cyclic guanosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
C_{max}	Maximum plasma concentration
COPD	Chronic obstructive pulmonary disease
CVD	Cardio vascular disease
CYP	Cytochrome
DMSO	Dimethyl sulfoxide
EAD	Early afterdepolarizations
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FSC	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIK	Gucose, insulin and potassium
GST	Glutathione-S-transferase
HBSS	Hank's Balanced Salt Solution
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
IFN- ɣ	Interferon-gamma
IHD	Ischaemic heart disease

IL-1ß	Interleukin 1 beta
I_{Na}	Invart sodium
LD/MS	Liquid chromatography–mass spectrometry
LDH	Lactat dehydrogenase
LDL	Low-density lipoprotein
M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
M199	Minimum essential medium
VCAM-1	Vascular cell adhesion molecule-1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PSFG	Penicillin, Streptomycin, Fluconazol, L- Glutamine
PVD	Peripheral vascular disease
PMN	Polymorphonuclear neutrophils
SR	Scavender receptor
TDR	Transmural dispersion of repolarization
TEMS	Trimetazidine European Multicenter Study
TF	Tissue factor
TGF-ß	Transforming growth factor beta
TLF	Toll-like receptors
TNF- α	Tumor necrosis factor alpha
QOL	Quality of life
2HCL	Dihydrochlore salt
3-KAT	Acetyl-CoA C-acyltransferase

9.2. List of tables

9.3. List of figures