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REVIEW ARTICLE

Myocardial infarction and oxidative damage in animal models: objective and expectations from the application of cysteine derivatives

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ABSTRACT

Reactive oxygen species (ROS) and associated oxidative stress are the main contributors to pathophysiological changes following myocardial infarction (MI), which is the principal cause of death from cardiovascular disease. The glutathione (GSH)/glutathione peroxidase (GPx) system appears to be the main and most active cardiac antioxidant mechanism. Hence, enhancement of the myocardial GSH system might have protective effects in the setting of MI. It follows that by increasing antioxidant capacity, the heart will be able to reduce the damage associated with MI and even prevent/weaken the occurrence of oxidative stress, which is highly ranked among the factors responsible for the occurrence of acute MI. For these reasons, the primary goal of future investigations should be to address the effects of different antioxidative compounds and especially cysteine derivatives like *N*-acetyl cysteine (NAC) and *L*-2-oxothiazolidine-4-carboxylic acid (OTC) as precursors responsible for the enhancement of the GSH-related antioxidant system's capacity. It is assumed that this will lay down the basis for elucidation of the mechanisms throughout which applicable doses of OTC will manifest a potentially positive impact in the reduction of adverse effects of acute MI. The inclusion of OTC in the models for prediction of the distribution of oxygen in infarcted animal hearts can help to upgrade existing computational models. Such a model would be based on computational geometries of the heart, but the inclusion of biochemical redox features in addition to angiogenic therapy, despite improvement of the post-infarcted oxygenated outcome could enhance the accuracy of the predictive values of oxygenation.

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Introduction

Cardiovascular disease is the number one cause of death globally (Lopez and Murray 1998). The incidence of cardiovascular disease is alarmingly high in industrialized countries, while over the years in developing countries it is showing constant growth (Lopez and Murray 1998; Towbin 2001), confirmed by 17.9 million deaths worldwide in 2016, and this number is expected to reach up to 23.6 million deaths annually by 2030 (Towbin 2001).

Myocardial infarction (MI) is a leading cause of morbidity in developed countries, despite the rapid progress in strategy for the treatment of myocardial ischemia (Steffens et al. 2009). MI is a significant acute disease of the myocardium caused by an imbalance between the heart muscle's need for oxygen and the supply of oxygen to the tissue; which leads to myocardial ischemia, degeneration of cardiomyocytes, irreversible damage to the heart, or even death (Wei et al. 2017). Previous studies have shown that oxidative stress caused by the production of reactive oxygen species (ROS), during cardiac damage induced by ischemia, played a key role in the development of MI (Wong et al. 2017). Oxidative stress is a pathological process in which the balance between the oxidative and antioxidative systems is disrupted, and ROS are produced to a large extent at the expense of the endogenous antioxidant capacity (Freitas et al. 2014).

It is also known that acute MI is a complex phenomenon that affects the mechanical, electrical, structural, and biochemical characteristics of the heart (Ruiz Petrich et al. 1996). Despite this complexity, impressive progress has been made in the understanding of the cellular processes associated with cardiac dysfunction and heart attack, and more importantly, in the application of this knowledge to therapeutic intervention (Kumar and Anandan 2007).

Catecholamine-induced myocardial damage

Catecholamines released by the adrenal medulla and the sympathetic central nervous system act as hormones and neurotransmitters that play an important regulatory role in the cardiovascular system (Costa et al. 2011). Cellular responses to catecholamines are mediated by two main types (α and β) of adrenergic receptors bound to G-proteins (Costa et al. 2011). α-adrenoceptors are divided into α1 (α1A,

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α1B, and α1D) and α2 (α2A, α2B, and α2C) subtypes, whereas β-adrenoceptors include β1, β2, and β3 subtypes (Alexander et al. 2011). α1 receptors bound to G-proteins are involved in the activation of phospholipase C, promoting an increase in the levels of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), through the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) (Costa et al. 2011). In cardiomyocytes, α1A and α1B are adrenoceptors that mediate positive inotropic effects and hypertrophic responses, while in vascular smooth muscle cells α1D adrenoceptors elicit arterial vasoconstriction, especially in the coronary arteries (Costa et al. 2011). α2 receptors are associated with inhibitory G-proteins (G_i and G_0) and participate in signal inhibition *via* adenylyl cyclase (AC) (Costa et al. 2011). β1 and β2 receptors participate in the activation of the Gs-AC-cAMP-protein kinase A (PKA) cascade, leading to an increase in $[Ca^{2+}]_i$. In the heart, they promote a positive ionotropic and/or chronotropic degree of excitability (bathmotropic) and lusitropic (level of muscle relaxation) effect, increasing the pulse rate, the volume of blood pumped from the heart, the volume of blood flowing from the atria, as well as relaxation of the heart muscle (mainly throughout β1 receptors) (Costa et al. 2011). In vascular smooth muscles, both β1 and β2 receptors mediate relaxation and vasodilation (Chruscinski et al. 2001). β3 receptors expressed by cardiomyocytes initiate a G_i-NO-cGMP signaling pathway, which has negative effects on the contractility of the heart (Gauthier et al. 2000). There are also five types of dopaminergic receptors (D1–D5). Only D1 and D4 are expressed in the heart, mediating inotropic effects.

Low-catecholamine concentrations are important regulators of myocardial contractility and its metabolism, but high levels of circulating catecholamines occur in conditions of excessive endogenous release or exogenous administration (Upaganlawa et al. 2010). High catecholamine concentrations cause stress on the myocardium, and destroy the energy reserves of the cardiomyocytes, leading to complex biochemical and structural changes that result in irreversible cellular damage and death (Liaudet et al. 2014). During an imbalance between oxygen demand and oxygen delivery to the heart, caused by prolonged activation of adrenergic receptors, catecholamines can destroy cardiomyocytes as a consequence of mitochondrial dysfunction, through two main mechanisms. The first one involves the accumulation of Ca^{2+}, sequential β-adrenergic activation of PKA, and subsequent phosphorylation of almost all Ca^{2+}-dependent proteins. The second mechanism is the occurrence of oxidative stress, primarily related to the transformation of catecholamines into 'monochromes', which are further subject to mitochondrial redox reactions, followed by a generation of large amounts of ROS. Hence, the accumulation of Ca^{2+} together with oxidative stress leads to increased mitochondrial permeability and cardiomyocyte death *via* both apoptosis and/or necrosis (Ferrari et al. 1991). ROS-induced cell death can initiate local inflammatory responses that are responsible for additional tissue damage mediated by oxidative stress (Panda et al. 2017).

To examine the cardio-protective effects of different compounds, a widely used and well-standardized experimental model of MI induced by isoproterenol (ISO) was established. The model was based on the severe stress of the myocardium induced by ISO, resulting in MI (Brooks and Conrad 2009). The induction of MI can also be performed by surgical procedures, such as aortic ligation, infusion of β-adrenergic agonists through implanted osmotic mini-pumps, as well as by coronary artery ligation. All these procedures are characterized by a high incidence of morbidity and mortality, not only because of the nature of the actions but also as a result of consecutive postoperative infections and complications (Lobo Filho et al. 2011; Shukla et al. 2015). Administration of ISO to animals provides a fast, simple, and noninvasive method for induction of cardiac damage similar to acute MI in humans (Shukla et al. 2015). The low mortality rate, and the high reproducibility and validity of the method in comparison to other animal models, makes it suitable for evaluating the effects of numerous cardioprotective agents in conditions of induced acute MI (Shukla et al. 2015).

ISO induced MI

ISO (1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride), is a synthetic catecholamine and a nonselective β-adrenergic agonist (Hadzi-Petrushev et al. 2011; Lalitha et al. 2013). In a low dose, it can be used in conditions of heart block and cardiac arrest. High doses or long-term administration of ISO leads to irreversible damage to the myocardium and myocardial necrosis (Lalitha et al. 2013). Administration of ISO induces characteristic myocardial damage in the subendocardial layer of the left ventricle followed by acute extensive myofibril degeneration (Zhang et al. 2008; Hadzi-Petrushev et al. 2018). Excessive stimulation of beta-adrenergic receptors with ISO disrupts the balance between the heart muscle's needs and the supply of oxygen, and that can lead to pathological changes in the myocardium (Allawadhi et al. 2018).

The main mechanisms of ISO-induced heart damage are complex and multifactorial, but the main damage from treatment with ISO includes the production of cytotoxic free radicals in the cardiomyocytes, followed by oxidative stress, and lipid peroxidation (Hadzi-Petrushev et al. 2018); leading to progressive damage of the mitochondria, production of inflammatory cytokines, an ionic imbalance with intracellular Ca^{2+} accumulation and heart damage (Figure 1) (Allawadhi et al. 2018).

The excessive stimulation of the adrenergic receptors causes increased myocardial contractility and increased heart rate, followed by a secondary increase in the oxygen demand that may exceed the supply of oxygen; thus creating areas of 'functional' hypoxia that may be potentiated by vasoconstriction in the coronary macro- and microcirculation, which can lead to a reduction in the supply of high-energy phosphates (Rona 1985). ISO can cause an increase in the heart rate, with doses that may cause lesions in the heart and lead to a drop in blood pressure. The drop in blood pressure is so pronounced that it can completely reduce coronary blood flow. It is assumed that necrotic lesions are ischemic infarct areas due to decreased blood flow during

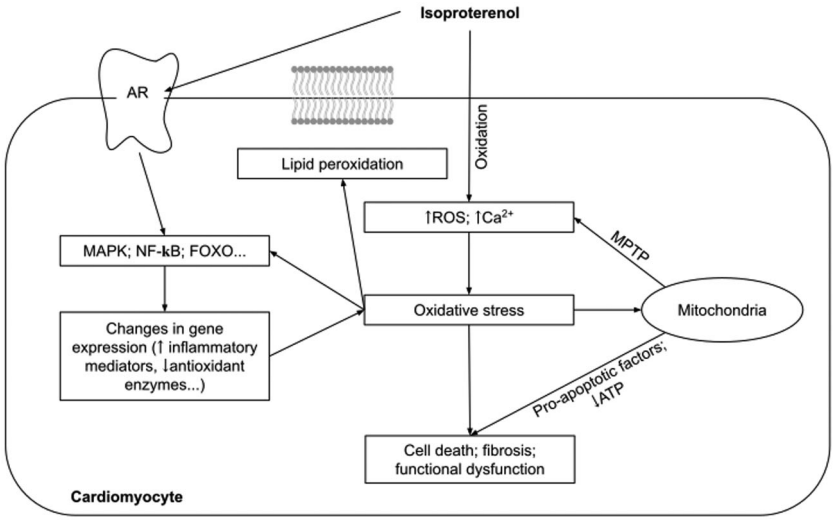

Figure 1. Schematic representation of the molecular mechanisms of isoproterenol (ISO)-induced heart damage. Isoproterenol causes harmful changes in heart tissue through oxidative stress and a decrease in the activity of antioxidant enzymes. ISO also causes a reduction in oxygen supply, leading to hypoxia in the heart followed by necrosis of the heart tissue. Increased lipid peroxidation causes membrane permeability and promotes cardiac hypertrophy. Phospholipase activation and inflammation cause acute heart injury and myocardial ischemia. Additionally, various signaling pathways, such as NF-κB, and mitogen-activated protein kinases (MAPK), such as p38, contribute to cell death. All of these changes sum up the typical features of ISO-induced damage, which if persisted for a long time, can lead to myocardial fibrosis. CVD: cardiovascular disease; ECG: electrocardiogram; ERK: extracellularly regulated kinase; JNK: c-Jun NH2-terminal kinase; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF-κB: nuclear factor-kappa-beta; ROS: reactive oxygen compounds (modified from Costa et al. (2011)).

the processes following enlarged amplitude and frequency of the heart contractions (Borkowski et al. 2011).

Changes in membrane permeability can lead to electrolyte imbalance (hypokalemia and hypomagnesemia with reduced intracellular Mg^{2+}), and an imbalance in other cellular homeostatic processes that contribute to additional myocardial damage (Bers and Despa 2009). The intracellular and mitochondrial Ca^{2+} increase as a response to β-adrenergic stimulation and activation of PKA results in more potent phosphorylation of almost all intracellular Ca^{2+}-dependent proteins, including *L-type* Ca^{2+} channels, phospholamban, and ryanodine receptor Ca^{2+} releasing channel (RyR2) in the sarcoplasmic reticulum (SR) (Zhang et al. 2013). PKA-dependent phosphorylation of the troponin (Tn) and myosin binding protein C leads to an increase in the $[Ca^{2+}]_i$, reducing the affinity of myofilaments for Ca^{2+} (Zhang et al. 2013). Persistent activation of β-adrenoceptors promotes activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMCII) via PKA-dependent and PKA-independent mechanisms (Swaminathan et al. 2012). Activated CaMCII phosphorylates different proteins, voltage-dependent Ca^{2+} channels, RyR2 Ca^{2+} release channels, and voltage-dependent Na^+ channels, which increases the $[Ca^{2+}]_i$ (Tokgözoğlu 2009). The increased content of Ca^{2+} in the myocardium during reperfusion results in excessive stimulation of the myofilaments, an increase in contraction forces, and oxygen demand, followed by increased adenosine triphosphate (ATP) depletion. The whole process is known as hyper contraction during acute MI (Nirdlinger and Bramante 1974).

The intracellular accumulation of Ca^{2+} induced by ISO also affects the membrane by activating Ca^{2+}-dependent phospholipases and proteases following the breakdown of high-energy phosphates (Ramos et al. 1984). Activation of Ca^{2+}-dependent enzymes further causes inhibition of membrane-bounded enzymes such as *Na^+/K^+- ATPases*, leading to an increase in the Na^+ levels and a loss of cytoplasmic K^+ ions. An increase in the concentration of Na^+ leads to the accumulation of Ca^{2+} through the *Na^+/Ca^{2+}* exchanger. In total, these changes lead to cellular dysfunction and cardiac damage (Ramos et al. 1984). As a result of the increased levels in $[Ca^{2+}]_i$, there is a progressive increase of Ca^{2+} in the mitochondria ($[Ca^{2+}]_m$), leading to rapid changes in the permeability of the inner mitochondrial membrane, accompanied by mitochondrial matrix edema, disruption in the control of cellular respiration and generation of ROS, which contributes to further deterioration of the Ca^{2+}-induced mitochondrial dysfunction (Khan et al. 2012). ''Fleckenstein's hypothesis'' for Ca^{2+}-mediated myocardial damage mediated by the effects of catecholamines is now described as the mitochondria-centric signal - transducer - effector (MSTE) signal path: Ca^{2+} accumulation in the mitochondria (*signal*) leads to oxidative stress in the mitochondria (*transducer*) and increased mitochondrial permeability (*effector*), which eventually leads to cell death via apoptosis and necrosis (Khan et al. 2012).

The pathophysiological changes activated by the rise in $[Ca^{2+}]_m$ are significantly amplified by the oxidative stress that occurs during prolonged exposure to high levels of catecholamines. First, during monoamino-oxidase (MAO) dependent oxidative deamination of the catecholamine, hydrogen peroxide (H_2O_2) is formed, and it can be converted to a highly reactive hydroxyl radical (OH·) through metal-mediated catalysis (*Fenton reaction*) (Behonick et al. 2001). Despite the various mechanisms proposed to explain ISO-induced heart damage, one of the important factors is the generation of highly cytotoxic free radicals by auto-oxidation of the catecholamine (Behonick et al. 2001). Different studies have shown that catecholamines immediately oxidize into toxic compounds called 'aminochromes'. This process happens spontaneously (auto-oxidation) with a low rate, but can

be significantly accelerated in the presence of oxidants and free radicals such as $O_2^{\cdot-}$, redox metals (especially iron and copper), and under the influence of enzyme-catalyzed reactions (especially of xanthine oxidase (XO), myeloperoxidase, and cytochrome oxidase) (Remiao et al. 2001). The oxidation of catecholamines is a transferring process of two electrons in which ortho-quinone derivatives are formed, followed by cyclization to leukoaminochromes, which are further oxidized to aminochromes (Taam et al. 1986). Aminochromes show direct adverse effects on the coronary arteries (vasoconstriction), while in the myocardium they cause inhibition of the oxidative phosphorylation and ability to bind Ca^{2+}, which together results in reduced force of contraction and *ex vivo* extensive necrotic damage (Taam et al. 1986). Besides their direct harmful effect, aminochromes may also induce the formation of large amounts of ROS through the redox processes that primarily occur in the mitochondria (Bindoli et al. 1990). The first step in this cycle is the reduction of aminochrome semiquinone by one electron, in the presence of nicotine adenine dinucleotide (NADH), catalyzed by complex I from the respiratory chain (Bindoli et al. 1990). In the second step, the semiquinone is instantly regenerated by the native aminochrome through auto-oxidation in the presence of oxygen, ended by the releasing of one molecule of $O_2^{\cdot-}$. Then the cycle is renewed via an enormous production of $O_2^{\cdot-}$ leading to over-oxidation of the catecholamines to their corresponding aminochromes, whose concentration increases exponentially (Liaudet et al. 2009). When the production of free radicals and oxidants overwhelms the endogenous antioxidant capacity, the state of oxidative stress develops with profound cytotoxic consequences, associated with oxidative damage of the lipids, proteins, and nucleic acids (Izem-Meziane et al. 2012).

Oxidative stress caused by catecholamines, in connection with significant accumulation of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, contributes to the opening of permeable pores in the cardiac mitochondrial membrane [mitochondrial permeability transition pore (mPTP)], well described in the ISO-induced cardiac damage model (Halestrap 2009; Izem-Meziane et al. 2012). Electro-physiologically, mPTP works as a nonspecific voltage-independent mega-channel, extending throughout both mitochondrial membranes. The opening of mPTP results in mitochondrial swelling, reduction of the mitochondrial membrane potential, and discontinuation in oxidative phosphorylation (Biary et al. 2011). This contributes to the second generation of free radicals via the process assigned as ROS-induced ROS-release (RIRR), which is responsible for amplification of the mitochondrial oxidation and opening of the mPTP, leading to permeabilization of the outer mitochondrial membrane and efflux of pro-apoptotic molecules (Brenner and Moulin 2012). Depending on the degree of openness of mPTP, according to Halestrap (2009) and Brenner and Moulin (2012), there are three possible scenarios in which cells can either: resist (as far as they are minimally open); die through the process of apoptosis (as far as they are moderately open); or necrotize (as far as they are massively, irresistibly open) (Decker et al. 1977).

ISO also causes an increase in the activity of lysosomal enzymes (Öllinger and Brunk 1995). Lysosomes represent a group of cytoplasmic organelles present in animal tissues that contain hydrolytic enzymes capable of the degradation of cellular constituents. Additionally, lysosomes play a major role in the processes of secretion and transport. The release of lysosomal enzymes in the intracellular medium and the level of sequential extra-lysosomal activity play a significant role in the progressive myocardial modification from a state of reversible myocardial ischemia to irreversible changes characteristic for acute MI (Halliwell and Gutteridge 1989). The lysosomal membrane is rich in phospholipids, which are a potent site for attack by free radicals. The lysosomal leakage may initiate apoptosis induced by oxidative stress (Halliwell and Gutteridge 2007).

Lack of oxygen and glucose results in impaired integrity of the cellular membrane, which can increase its permeability, and thus allow enzymes, like serum glutamate-pyruvate transaminase (SGOT), troponin I (TnI), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK-MB) to exit from the cell (Mendez et al. 2005). The level of markers of cardiac damage [aspartate transaminase (AST), TnI, LDH, creatine kinase (CK-MB), CPK-MB, serum glutamate-oxaloacetate (SGO) and SGOT, and proinflammatory cytokines – C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α)] are amplified in response to ISO, proportional to the level of necrosis (Dröge 2002).

In summary, long-term high levels of catecholamines can cause huge myocardial damage that leads to morphological alterations similar to those caused by MI, involving varying degrees of necrosis and apoptosis of cardiomyocytes, infiltration of polymorphonuclear and mononuclear leukocytes, edema in the interstitium, subendocardial and subpericardial hemorrhages, and progressively developing regions with fibrous tissue. From an ultrastructural point of view, the main alterations of catecholamine-induced heart damage are caused by myofibril damage, mitochondrial swelling, and dilatation of the SR (Rona 1985).

Reactive species and oxidative stress

Molecular oxygen as a terminal electron acceptor plays a very important role in many metabolic processes associated with the existence of aerobic organisms. ROS are products of the partial reduction of molecular oxygen (Figure 2). Typically, molecular oxygen is reduced by four electrons in the mitochondrial electron transport chain, resulting in water formation. With the addition of one electron, molecular oxygen is converted to a superoxide anion, which is further reduced to H_2O_2, and subsequently to hydroxyl radicals. The reaction is completed by the formation of water as a result of the addition of an electron and a proton to the hydroxyl radical (Bolli et al. 1989).

Small amounts of oxygen (between 0.4 and 4% of the total amount of used oxygen) are reduced to O_2^- *via* the electron transport chain in the mitochondria during normal oxidative phosphorylation, as an essential process for ATP generation. Subsequently, O_2^- can be converted to other

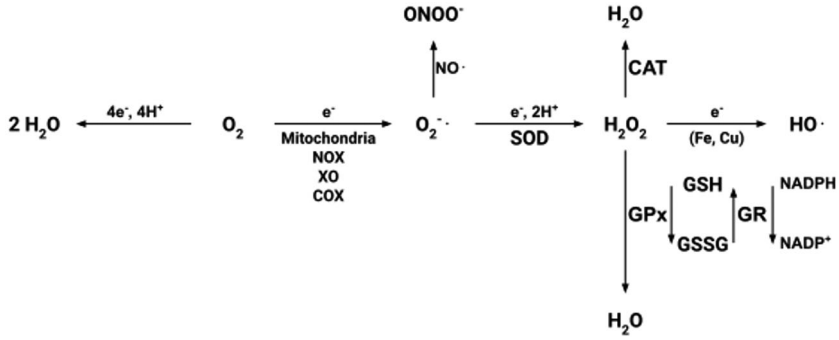

Figure 2. Relationships between different ROS [formation of ROS and RNS under the action of different stimuli (modified from Halliwell and Gutteridge (2007))].

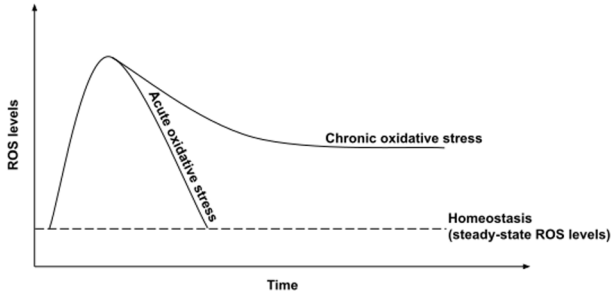

Figure 3. Graphic representation of the dynamics of the creation of reactive oxygen forms in living organisms (modified from Lushchak (2011)).

ROS and reactive nitrogen compounds (RNS) (Figure 3) (Venardos et al. 2007). In normal physiological conditions, O_2^- is rapidly converted to H_2O_2 by a key mitochondrial enzyme, manganese-dependent superoxide dismutase (Mn-SOD), and by cytosolic, copper, and zinc-dependent superoxide dismutase (CuZn-SOD) (Guzik et al. 2002). H_2O_2 is not a free radical but is considered to be a ROS because it is very reactive. H_2O_2 is converted to H_2O and O_2 by glutathione peroxidase (GPx) (in the mitochondria), or diffuses into the cytosol where it is degraded under the action of catalase (CAT) from peroxisomes. In addition, H_2O_2 can also be converted to a highly reactive hydroxyl radical (HO^-) in the presence of a reducing agent, such as metals – Cu or Fe (*Fenton reaction*) (Teixeira et al. 1999). The high reactivity of $OH^·$ is justified by the fact that it reacts with the first molecule it comes in contact with (such as unsaturated fatty acids) resulting in lipid peroxidation and cell membrane disruption (Cilento and Nascimento 1993). The production of one molecule of ROS can lead to the production of many others by a chain reaction of free radicals. As summarized in Figure 3, $O_2^·$ can be produced via the univalent reduction of oxygen by several different oxidases including NAD(P)H oxidase, XO, cyclooxygenase, and endothelial nitric oxide synthase (eNOS) (Guzik et al. 2002). Singlet oxygen ($^1O_2$), an electronically excited state of molecular oxygen, is one of its very reactive and toxic forms. $^1O_2$ is not a radical and has an electrophilic character. Accordingly, $^1O_2$ can induce oxidative reactions with organic components in the parts of their molecules that are rich in electrons (Teixeira et al. 1999). The high reactivity of the $^1O_2$ with biological macromolecules makes it a potential attacker when it is produced in the cell. This is especially visible through its ability to damage guanine components of nucleic acids, causing toxic and mutagenic effects (Cilento and Nascimento 1993; Turko et al. 2001; Ferrari et al. 2004; Lushchak 2011; Rodrigo, Libuy, et al. 2013; Rodrigo, Prieto, et al. 2013; van der Pol et al. 2019). Mechanisms for enzymatic formation of $^1O_2$ are explained in several cases as a part of the immunological defense in inflammatory responses. It is believed that different cell types, such as eosinophils, macrophages, and neutrophils can generate $^1O_2$ in response to inflammation (Penna et al. 2009).

The reactive nitrogen species include free radicals, such as nitric oxide (NO^-), nitrogen dioxide ($NO_2^{·-}$), and peroxynitrite ($ONOO^-$), which do not belong to the group of free radicals. $NO^·$, known as an endothelium-derived relaxing factor (EDRF), generated from *L*-arginine by eNOS activity, is considered to be a protective molecule of the vascular system (Wang and Zweier 1996). In addition, $NO^·$ easily reacts with O_2^-, producing a highly reactive molecule of $ONOO^-$. Hence, variations in the production of NO^- and O_2^- by the endothelium are one of the mechanisms responsible for the regulation of vascular tone and blood pressure. However, the partial pressure of O_2 in the body is reduced to the level of comfortable metabolic limit, and while the ionic concentrations of free Fe^{2+} and Cu^+ are strictly controlled, the formation of ROS cannot be eliminated. Their uncontrolled generation loads the antioxidant capacity of the cell, which results in damage and oxidation of the lipids, proteins, nucleic acids, and various transcription factors (Rodrigo, Libuy, et al. 2013; Rodrigo, Prieto, et al. 2013).

Taking into account that in our bodies, ROS are continuously generated and eliminated, their concentration is a dynamic parameter, or in other words, the concentration of ROS is found to be in a steady-state condition. This means that the degree of generation is in some way equal to the degree of elimination. However, the concentration of ROS may change for various reasons, leading to a violation of the redox status known as oxidative stress (Figure 4). Oxidative stress is a condition that arises when the concentration of ROS, acute or chronic, induces disruption in the cellular metabolism and its regulation and causes damage to the cellular components. If the concentration of ROS is increased while the antioxidant capacity is strong enough to maintain balance *via* the elimination of ROS, the level of ROS will quickly return to its initial state. Such a short-lived increase in ROS is called acute oxidative stress. However, in cases when the efficiency of the antioxidant system is not strong enough to cause a reduction in ROS concentrations to the

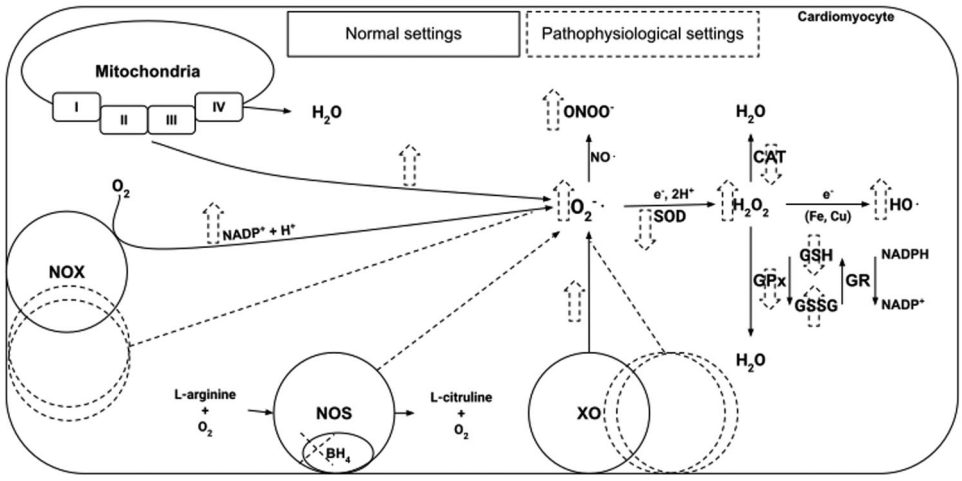

Figure 4. Oxidative stress and antioxidant defense mechanisms in cardiomyocytes under physiological and pathological conditions. In physiological conditions, oxidative stress is represented in the form of reactive oxygen forms generated in small quantities by the electron transport chain in mitochondria, NADPH oxidase (NOX), xanthine oxidase (XO), and nitric oxide synthase (NOS). In pathophysiological conditions, the increased oxidative stress is due to the increased expression of NOX and XO, together with the blocking of the electron transport chain in the mitochondria and the cleavage of NOS. Expression and activity (dashed lines) of SOD, CAT, and GPx are reduced. GSH levels are also reduced, while GSSG levels are elevated. This severe increase in oxidative stress eventually leads to hypertrophy, fibrosis, apoptosis, and myocardial contractile dysfunction (modified from Ferrari et al. (2004)).

level of the initial steady-state, and elevated ROS levels are maintained for a long time, this situation is assigned as chronic oxidative stress. In this situation, only an increase in the effectiveness of the antioxidant system can restore the equilibrium concentration of ROS to the initial level. In certain circumstances, when the affected system cannot return to the initial level, the ROS concentration is stabilized at a higher level, which is referred to as a quasi-stationary level (Ferrari et al. 1993).

Sources of free radicals and oxidative damage to the myocardium

Advances in cardiovascular research have identified oxidative stress as an important pathophysiological process in the development and progression of heart disease (Grace 1994).

By definition, myocardial ischemia is a condition that occurs when the delivery of oxygen is insufficient to meet the oxidative needs of the mitochondria. The fact that there is a lack of oxygen availability during ischemia does not mean that free oxygen radicals cannot be formed. Conversely, the changes occurring in the metabolism during ischemia may be responsible for the formation of residual oxygen free radicals (Murphy 2009). By returning to a normal level, blood flow increases the level of oxygenation of the heart tissue and initiates an explosion in the production of ROS (Murphy 2009). ROS are major initiators of myocardial damage during reperfusion, a phenomenon called myocardial reperfusion injury (MRI) (Friedl et al. 1990).

Several potential sources of free radicals in the myocardium have been described (Kehrer et al. 1987). They include mitochondria, cell membranes, and endothelial cells (Granger 1988), but the mitochondria are the most important sites where free oxygen radicals are formed (Wang et al. 2012).

During ischemia, due to lack of oxygen, the electron transport chain may not function properly and therefore high levels of ROS are produced. Adenine nucleotide reserves are partially depleted, so the protein carriers in the mitochondria are in a completely inactive state (Venardos et al. 2007). These conditions can lead to a state where more electrons will leave the respiratory chain and in turn will react with the residual molecular oxygen trapped inside the mitochondrial membrane, which will result in the formation of superoxide radicals. Re-oxygenation by reperfusion will return energy to the mitochondria, but still, the action of electrons through cytochrome oxidase can be reduced, due to ADP deficiency. As a result, the number of electrons that will leave the respiratory chain will increase and they have available molecular oxygen to react with (Kloner et al. 1989; Ferrari et al. 1998) (Figure 5).

XO is predominantly present in the vascular endothelium in the normal physiological state of the heart and participates in the production of $O_2^{·-}$, H_2O_2, and $OH^·$ as ancillary products of its normal metabolic activity (Friedl et al. 1990). In pathological conditions, such as hypoxia or ischemia, xanthine dehydrogenase may be converted into XO (Kehrer et al. 1987; Granger 1988). At the same time, ATP is degraded to hypoxanthine, which is accumulated in the ischemic tissue. During reperfusion, in the presence of large amounts of molecular oxygen and a high concentration of hypoxanthine, an explosion in the production of $O_2^{·-}$ occurs (Wang et al. 2012). During ischemia/reperfusion this enzyme catalyzes the production of uric acid with ancillary production of $O_2^{·-}$ (Venardos et al. 2007). The release of superoxide anions results from the activation of neutrophil leukocytes and their adhesion to endothelial cells, which stimulates the formation of XO in endothelium with subsequent production of $O_2^{·-}$ (Ferrari et al. 1998).

Activated neutrophils during reperfusion are a potential source of ROS. The inflammatory response in ischemia/reperfusion causes the release of substances (chemo-attractants) that induce neutrophil infiltration and activation. Further, they adhere to injured endothelium where they initiate the production of ROS *via* NADPH oxidase (NOX) in their cell

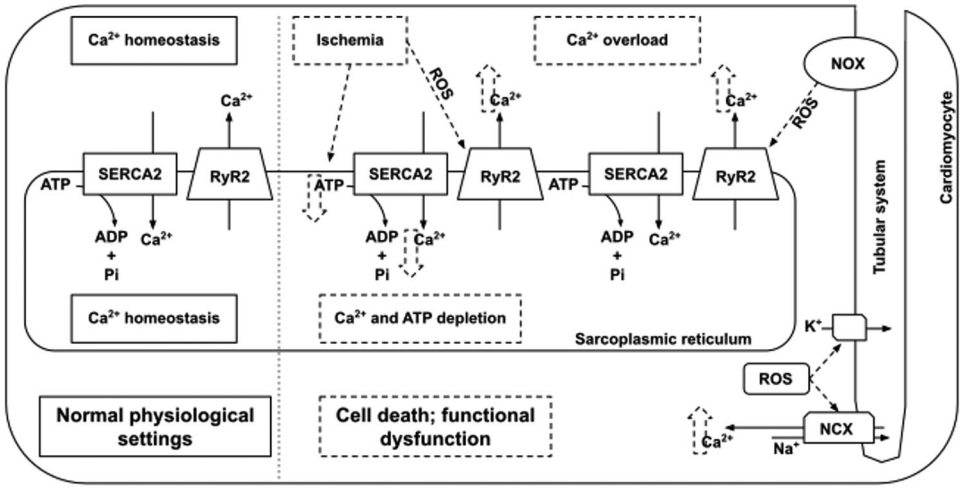

Figure 5. Effects of excessive oxidative stress on the myocardium. As a result of heart damage, the accumulation of ROS has detrimental effects on the myocardium. 1. The electrophysiology of cardiomyocytes is affected by elevated ROS levels. ROS in turn changes the function of Na^{2+}/Ca^{2+} exchanger (NCX), leading to Ca^{2+} infusion and Na^{+} efflux. ROS increases Ca^{2+} influx through L-type calcium channels. An increase in ROS also increases $sarcK_{ATP}$ currents, leading to a shortening of the action potential duration, while the reduction of the Kv currents and the increased delayed sodium currents lead to a prolongation of the action potential duration. 2. Excessive ROS production promotes ryanodine receptor (RyR2) activity and inhibits Ca^{2+}-adenosine triphosphatase 2 activity in the sarcoplasmic reticulum (SERCA2), resulting in calcium accumulation and reduced sensitivity of calcium myofilaments, which eventually leads to contractile dysfunction. 3. Mitochondria respond to ischemic injury by producing high levels of ROS, but the increased presence of ROS on the other hand results in further mitochondrial dysfunction and energy metabolism. 4. Increased ROS is also responsible for increased fibrosis due to increased metalloproteinase inhibitors tissue inhibitor of matrix metalloproteinase (TIMP) and reduction of matrix metalloproteinases (MMPs) expression (modified from van der Pol et al. (2019)).

membranes, which reduces molecular oxygen to superoxide anion ($O_2^{\cdot -}$) using NADPH as an electron source (Wang et al. 2012). This enzyme is largely present in activated neutrophils where it generates huge amounts of toxic $O_2^{\cdot -}$ and other important ROS for their bactericidal function (Romson et al. 1983; Engler et al. 1986). Activated neutrophils cause tissue injuries by releasing additional oxidants (myeloperoxidase) and hydrolytic enzymes (elastase, collagenase, cathepsins, and hyaluronidase) (Ferrari et al. 1993). The phenomenon of capillary occlusion with neutrophils can reduce blood circulation further, contributing to exacerbation of the condition of ischemia (Ferrari et al. 1993). Increased capillary permeability as a consequence of superoxide anions can cause edema and an increase in interstitial pressure, leading to reduced local blood circulation. Whether neutrophils will represent a major source of free radicals during ischemia/reperfusion strictly depends on how early they infiltrate the tissue. It has been proven that in MI neutrophils content increases 17-fold after 24 h (Grace 1994).

During ischemia, the superoxide anion may also be produced during the metabolism of arachidonic acid via the pathway of cyclooxygenases (Ferrari et al. 1993). Studies have shown that the accumulation of Ca^{2+} during ischemia/reperfusion may activate phospholipases that participate in the degradation of phospholipids from the cell membrane, thereby releasing arachidonic acid. It is further metabolized by cyclooxygenase and lipoxygenase activities to prostaglandins and leukotrienes. These metabolic pathways involve electron transfer that can initiate the formation of free radicals (Singal et al. 1983).

Oxidative stress can induce functional changes, including disruption in the activity of Ca^{2+}-ATP-ase in the sarcoplasmic reticulum (SERCA), which causes inhibition in the uptake of Ca^{2+} from the cytoplasm and subsequent disruption of the sodium-calcium exchanger (NCX) activity that increases the influx of Ca^{2+}, while oxidized myofilaments manifest reduced sensitivity to Ca^{2+} (Dixon et al. 1992). ROS causes an alteration in the function of the *Na^{+}/Ca^{2+}* exchanger (NCX), leading to Ca^{2+} influx and Na^{+} efflux. ROS also increases Ca^{2+} influx through *L-type Ca^{2+} channels*. Accumulation of Ca^{2+} and reduction in the myofilament's sensitivity to Ca^{2+} may eventually lead to contractile dysfunction (Dixon et al. 1992). Stagnation in the regulatory mechanisms for Ca^{2+} under the action of ROS ultimately leads to the accumulation of Ca^{2+} in the intracellular space and cell death. Recently, it has also been proven that the function of RyR is controlled by ROS (Donoso et al. 2011). Prosser et al. (2011) have shown that NOX and RyR can be found in proximity to T-tubules of cardiomyocytes (Donoso et al. 2011). Accordingly, the increased production of ROS after reperfusion of the myocardium may lead to increased functioning of RyR, resulting in intracellular accumulation of Ca^{2+} (Takimoto and Kass 2007) and triggered activation of pro-apoptotic intracellular pathways, necrosis, and changes in the electrophysiology (Mitrokhin et al. 2019) and contractility of the cardiomyocyte's machinery (Mohora et al. 2009; Shim, Mitrokhin, Gorbacheva, et al. 2017; Shim, Mitrokhin, Kazanski, et al. 2017) (Figure 6).

Finally, ROS also show pro-fibrotic functions, inducing fibroblast proliferation in the heart and changes in the activity of matrix metalloproteinases (MMPs), which leads to extracellular remodeling (Takimoto and Kass 2007).

Redox regulation of NF-κB

ROS act not only through direct modification of organic molecules but are involved also in the regulation of the expression of certain genes (Kim et al. 2003). One of the most studied is the nuclear transcription factor-κB (NF-κB), as a corresponding factor to the changes in the oxidative status of the cell (Kim et al. 2003). NF-κB is a heterodimeric protein,

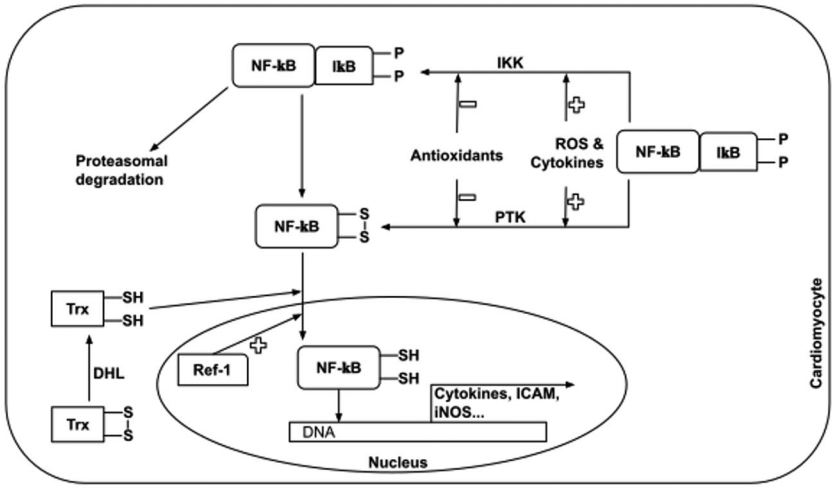

Figure 6. NF-kB signaling pathway. Exposure of cells to oxidative or proinflammatory stimuli causes activation of several kinases, such as IKK, which further activates NF-κB through phosphorylation and degradation of IkBα. Activated tyrosine kinase (PTK) can also phosphorylate tyrosine residues on IkBα translating this protein into degradation protease protein, but facilitating its dissociation from NF-κB. The free, activated NF-κB, in the form of the p65-p50 heterodimer, is transported to the nucleus, where it binds to the kB sequence located in the promoter region of the target genes. Thioredoxin (Trx) reduced by dihydrolipoic acid (DHL), and redox factor 1 (Ref-1) regulate the redox status of NF-kB in the nucleus. The thioredoxin is simultaneously transported to the nucleus upon activation of NF-κB and may physically interact with the p50 subunit before binding to DNA; Ub: ubiquitin (modified from Mohora et al. (2009)).

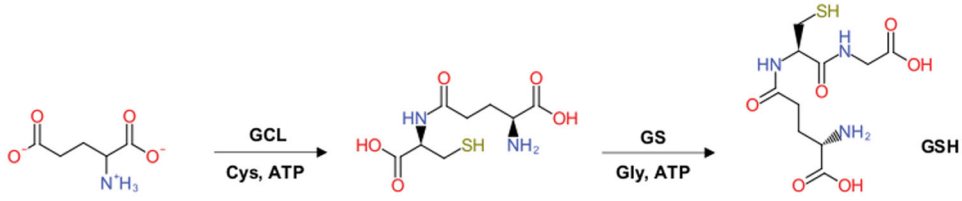

Figure 7. Biosynthesis of GSH (modified from Cacciatore et al. (2010)).

a ubiquitous redox-sensitive transcription factor involved in the control of several normal cellular processes such as immune and inflammatory responses, cell growth, and apoptosis (Chandra et al. 2000).

The ROS-regulated NF-κB activation induces transcription of protein mediators, such as proinflammatory cytokines that activate signaling pathways responsible for cellular death (Chen and Greene 2004). Thus, oxidative stress, ROS, and inflammation are closely interrelated in a single process. This phenomenon possesses important molecular bridges that are activated in the presence of ROS (Bowie and O'Neill 2000).

The NF-κB transcription factor contains cysteine residues in its structure (sensitive to redox changes), which ensure its interaction with specific binding regions in DNA. These interactions are achieved *via* 1) formation of hydrogen bonds between the free thiol groups of the transcription factor and the nitrogen bases of the DNA molecule, 2) intra- or intermolecular disulfide bonds (which ensure a favorable conformation adequate for interaction between the factor and DNA), and 3) cation coordination (especially zinc, which determines the formation of the zinc-finger motifs in the structure of the transcription factor, facilitating its interaction with DNA) (Sun and Oberley 1996; Pavlović et al. 2002). The DNA-bounded form of NF-κB is a heterodimeric protein consisting of p-65 and p-50 subunits, though there are other hetero- and homodimeric species (Siebenlist et al. 1994). In unstimulated cells, NF-κB exists as an inactive cytoplasmic complex with attached inhibitory κB (IκB) proteins. In terms of cellular compartmentalization, NF-κB transcriptional activation takes place in two separate steps. The first step is the degradation of IκB in the cytoplasm and subsequent translocation of the complex into the nucleus. The second step is DNA binding and initiation of transactivation in the nucleus (Hayden and Ghosh 2008). Extracellular stimuli, such as proinflammatory cytokines (TNF-α and IL-1β), ionizing radiation, ROS, and mitogens lead to activation of the IκB kinase complex (IKK), which causes phosphorylation of the two serine residues of IκB, responsible for IκB ubiquitination and consequential degradation in the proteasomes (Figure 7) (Cacciatore et al. 2010). IκB dissociation from the IκB-NF-κB complex allows translocation of the activated dimer p-50/p-65 into the nucleus, where it binds to an NF-κB element located in the promoter region of the targeted genes, controlling their expression (Kabe et al. 2005). NF-κB can also be activated independently by IκBα degradation, *via* phosphorylation of IκBα's tyrosine residues, which makes this protein resistant to degradation in proteasomes (Gallagher et al. 2007). In conditions where NF-κB is activated by ROS *via* phosphorylation of its inhibitory subunit, IκB promotes transcription of the genes involved in the inflammatory and profibrotic responses, such as IL-6, transforming growth factors β (TGF-β), and TNF-α (Opie et al. 2006; Liakopoulos et al. 2007). These molecules act in various tissues, especially in the heart, where they cause remodeling of the extracellular matrix and fibrosis (structural remodeling), which alters the electrophysiological characteristics of the heart (Kazanski

et al. 2017; Mitrokhin et al.2017). Some studies have shown that the process of NF-κB activation is associated with cardiac dysfunction, ventricular hypertrophy, and abnormal growth of the heart (Chunhua et al. 2017). It causes a higher generation of free radicals and activates the signal pathway *via* mitogen-activated protein kinase (MAPK). MAPK consists of three subfamilies – extracellularly regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 MAPK. In the heart, ischemia regulates pro-inflammatory and pro-apoptotic responses through JNK and p38 (Chunhua et al. 2017). The MAPK signal pathway additionally activates the inflammatory NF-κB signaling pathway that stimulates the production of inflammatory cytokines, e.g. TNF-α, IL-1, IL-6, etc., causing further damage to the myocardium (Mitrokhin et al. 2015a, 2015b; Ovchinnikov et al. 2015a, 2015b; Aksyonov et al. 2015; Filatova et al. 2019; Mitrokhin et al. 2021). Assuming that NF-κB, as a key mediator of the inflammatory processes in the cardiovascular system, is redox regulated, the strategic treatment aiming to increase the antioxidant defense should serve as a potential solution in the reduction of damage to the heart.

Antioxidant defense of the myocardium

In cardiomyocytes, as in many other cell types, the major endogenous components of the antioxidant defense system responsible for ROS inactivation are enzymes like SOD, CAT, GPx, and other endogenous antioxidants, such as ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), nicotinamide adenine dinucleotide (NAD^+), glutathione (GSH), carotenoids, coenzyme Q, and thioredoxins (Trx) (Neri et al. 2015).

NAD^+, together with its reduced dinucleotide NADH, represent key players in the oxidative reactions involved in the production of energy (Mericskay 2016). Aside from its role in the regulation of cellular energy metabolism, NAD^+ is a precursor of the phosphorylated pair of $NADP^+$/$NADPH^+$ dinucleotides, responsible for the detoxification of ROS (Mericskay 2016). In a few experimental models of mice with heart disease, different authors have found reductions in the myocardial levels of NAD^+ (Pillai et al. 2005). In different membranes, the liposoluble α-tocopherol acts as a 'scavenger' of the free radicals and interrupts the lipid chain peroxidation of polyunsaturated fatty acids (PUFAs) (Ferrari et al. 1983). α-tocopherol works in synergy with ascorbic acid, which reacts with α-tocopheroxy radicals and regenerates them into α-tocopherol, while produced ascorbic radicals are reduced back to ascorbate by NADPH reductase (Packer et al. 1979; Ferrari et al. 1983). In addition, ascorbic acid acts directly as an antioxidant in the cytosol and the extracellular fluid as well (Packer et al. 1979).

SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. In the mammalian heart, two forms of SOD have been found: in the cytosol, CuZn-SOD, and in the mitochondria, Mn-SOD (McCord and Fridvich 1969). The total SOD activity in the myocardium is similar among different mammals (Marklund et al. 1982). The CAT and GPx are also important enzymes in the metabolism of H_2O_2 generated by SOD (Roos et al. 1980). Their catalytic activity in the myocardium shows small variations among mammals (Janssen et al. 1993) and is 20 – 30 times less active than in the liver (Nohl and Jordan 1980). The CAT is predominantly membrane-bound in the peroxisomes of most tissues (Lawrence and Burk 1978), but in myocytes, significant CAT activity is found in the mitochondrial matrix (Lawrence and Burk 1978). GPx (a selenium-dependent enzyme) is present in significant concentrations in the myocardium (Curello et al. 1985). GPx utilizes the reducing agent NADPH in the reaction with GSSG (oxidized GSH) formed from GSH (reduced GSH), which is in dynamic equilibrium with the total sulfhydryl (SH) groups present in the cell. Disulfides derived from the reaction of GSSG with other thiol groups from different proteins, form an important part of the total GSH pool, which equilibrium is completely regulated by thiol transferases (Meister 1988). The activity of GPx is almost the same in guinea pigs and humans, but is 10 times higher in the rat heart, while the activity of GR and the levels of reduced and oxidized GSH show similarities in both species (Meister 1988). Also, there are pieces of evidence in the literature showing that GSH plays an important role in cardiac metabolism (Lu 1999).

Glutathione (GSH)

GSH (γ-L-glutamyl-L-cysteinyl glycine), an endogenous water-soluble tripeptide, is the most abundant representative of the thiol compounds in mammalian cells with concentrations up to 10 mmol/l (Townsend et al. 2003). The liver, which contains the highest GSH concentration, is the major tissue involved in the biosynthesis of GSH (Valencia et al. 2001). The intracellular GSH is stored in form of a reduced monomer (GSH), or as a disulfide dimer formed through its oxidation (GSSG), which usually accounts for less than 1% of the total intracellular GSH content. In the heart, GSH is a predominantly intracellular molecule with a concentration of up to 1.1 μmol/g. More than 95% of the GSH in the heart is in the reduced form, and the ratio between reduced and oxidized GSH in aerobic conditions is about 50:50 (Sies 1999). Additionally, an important fraction of the total intracellular GSH is represented by GSH in various forms of thioesters (Franco et al. 2007). GSH is 85 – 90% distributed freely in the cytosol, although it can be compartmentalized into various organelles, including mitochondria, peroxisomes, nuclear matrix, and endoplasmic reticulum (Anderson 1998). GSH is a tripeptide, with a small molecular weight, which contains glutamine, cysteine, and glycine. Due to the residues of cysteine, GSH is rapidly oxidized into GSSG by electrophilic compounds (e.g. free radicals, ROS, and reactive nitrogen species) (Griffith 1999). In the molecule of GSH, where the glutamine is bound to the cysteine, there is an unusual binding by amide bonds formed between the γ-carboxylate group and the amino group of the cysteine. This unusual γ-peptide bonding protects the tripeptide from degradation by aminopeptidases, and it can be hydrolyzed only by the enzyme γ-glutamyl transpeptidase (γ-GT) present on the surface of certain cells (Franco et al. 2007). In addition, the carboxyl terminus of glycine within GSH protects it from decomposition by the intracellular γ-glutamyl cyclo-transferase (GGT) (Kozak and Tate 1982). As a consequence, GSH is resistant to

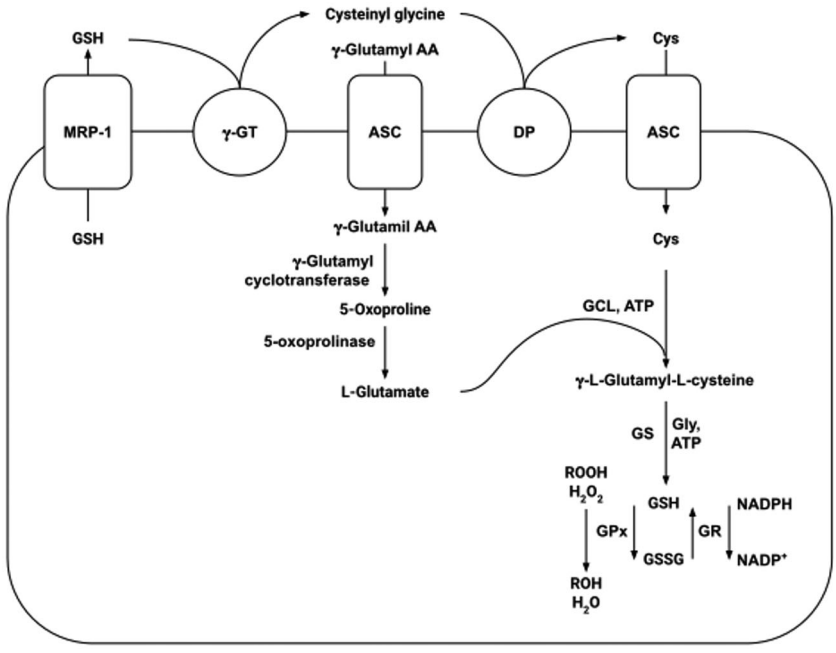

Figure 8. Biosynthesis and metabolism of glutathione (modified from Singh et al. (2012)).

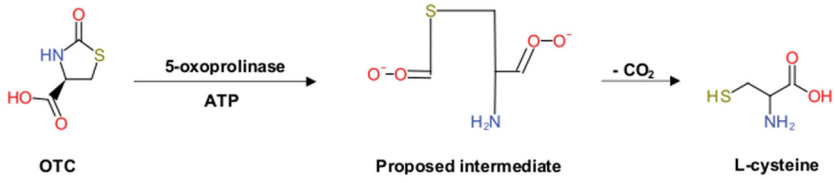

Figure 9. Formation of OTC-derived cysteine by the action of 5-oxoprolinase.

intracellular degradation and can be metabolized only extracellularly.

GSH biosynthesis and metabolism

GSH is synthesized in two consecutive ATP-dependent reactions catalyzed by cytosolic enzymes. In the first step, the enzyme glutamate-cysteine ligase (GCL), also called γ-glutamyl cysteine synthase (GCS), catalyzes the production of γ-glutamyl cysteine by the reaction which occurs between the γ-carboxyl group of *L*-glutamine and the amino group of *L*-cysteine (γ-peptide-binding). In the second reaction, catalyzed by the enzyme glutathione synthase (GS), also known as GSH synthetase, *L*-glycine is added by binding the amino residue from glycine to the carboxyl group of the cysteine from dipeptide γ-glutamyl cysteine, thereby forming GSH (Figure 8) (Wang and Ballatori 1998; Fang et al. 2002; Singh et al. 2012).

The balance between cellular synthesis and GSH utilization is regulated by the feedback inhibition of the GCS catalyzed reaction by its end product – GSH (Curello et al. 1987). The availability of cysteine is a limiting factor in the synthesis of GSH. Cysteine is extremely unstable in the extracellular fluid and rapidly auto-oxidizes into cystine, through a reaction that produces potentially toxic free oxygen radicals. The γ-glutamyl cycle allows efficient utilization of GSH as a reservoir of cysteine (Figure 9). GSH is released from the cells *via* carrier-mediated transporters, while the process of GSH degradation occurs in the extracellular compartments in which membrane-bounded gamma-glutamyl transpeptidase (γ-GT) is expressed. This heterodimer glycoprotein catalyzes the hydrolysis and transpeptidation of the γ-glutamyl group from GSH and GSH-conjugated compounds to other acceptors (amino acids and other dipeptides), which is the way cysteinyl glycine conjugates are released (Masella et al. 2005). These products are further hydrolyzed by dipeptidases to cysteine and glycine (Tandoğan and Ulusu 2006). Cysteine, together with the γ-glutamyl-amino acids, formed by the action of γ-GT is transported to the cells *via* a sodium-dependent neutral amino acid transport system (ACE). γ-glutamyl derivatives formed during this reaction represent substrates for the gamma-glutamyl cyclotransferase, which converts them to 5-oxoproline and corresponding amino acids. Finally, 5-oxoproline undergoes an ATP-dependent conversion to *L*-glutamate in a reaction catalyzed by the intracellular enzyme 5-oxoprolinase (Hiranruengchok and Harris 1995).

All of the above-mentioned reactions build up the so-called gamma-glutamyl cycle that provides amino acid precursors for additional GSH synthesis (Rodrigo, Libuy, et al. 2013; Rodrigo, Prieto, et al. 2013).

GSH redox cycle

GSH is involved in many biologically important processes. The key functional element in GSH is the SH group of

cysteine, which is involved in the reactions of reduction and conjugation as one of the most important functions of GSH (Rodrigo, Libuy, et al. 2013; Rodrigo, Prieto, et al. 2013). As an important antioxidant, GSH participates non-enzymatically and enzymatically in the fight against toxic compounds (Gil et al. 2018).

GSH is a key factor in the detoxification of electrophilic and reactive oxygen intermediates. As a determining factor of the SH/disulfide ratio (Shiomi et al. 2004), GSH modulates the activity of some enzymes and is also involved in the transport of amino acids across the cell membrane. In addition, GSH as a co-substrate of GPx plays an essential protective role in the fight against free oxygen radicals and protects membrane lipids from peroxidation, since the activity of SOD in the heart is four times lower than in the liver, while CAT activity is extremely low as well. This protective mechanism results in the formation of high levels of intracellular GSSG. Hence, changes in the cellular GSH status provide important information about cellular oxidative events and tissue accumulation and/or release of GSSG into the coronary circulation, which is a sensitive and accurate indicator of oxidative stress (Diguet et al. 2018).

GSH together with SOD, CAT, Trx reductase, and various intracellular redox systems, participates in the 'cleansing' of ROS and the protection of cells from oxidative stress, representing the first line of defense (Karlsen et al. 1981). GSH can directly destroy free radicals and peroxides accumulated in the cells during oxidative stress through the formation of mixed disulfides or via oxidation to GSSG. Additionally, GSH as a co-factor or substrate for various enzymes, together with these GSH-associated enzymes, provides a second line of defense. These redox reactions catalyzed by GPx and GR are some of the most important functions of GSH (Masella et al. 2005). GPx participates in the detoxification of hydroperoxides, such as H_2O_2, fatty acid hydroperoxides, and phospholipid hydroperoxides mediated by GSH, which acts as an electron donor in the reduction reaction. GSH is oxidized to GSSG, in the GPx-catalyzed reaction (Masella et al. 2005). GSSG has been proven to have the capability to react with protein SHs, forming mixed protein-GSH disulfides (Hiranruengchok and Harris 1995).

Maintaining the GSH/GSSG equilibrium in the cells is critical concerning cell survival, which means that the strict regulation of this system in some way is mandatory. Extreme levels of oxidative stress can increase GSSG levels immediately, at the expense of GSH. Therefore, to maintain a constant GSH level, the activity of the NADPH-dependent enzyme GR is required, which catalyzes the reaction of reduction of GSSG to GSH (Hiranruengchok and Harris 1995) (Figure 9). In the studies with ISO-induced cardiac hypertrophy, the level of production of free radicals is dramatically increased while the level of antioxidants, especially CAT, SOD, GSH, and GPx is significantly reduced (Hiranruengchok and Harris 1995; Masella et al. 2005). This means that in case of damage, the heart possesses a limited antioxidant capacity compared to the kidneys and liver, making it particularly sensitive to ROS (Masella et al. 2005). Therefore, therapeutic strategies using agents with strong antioxidative effects for targeting oxidative stress can be investigated for their protective function on MI.

Targeting of oxidative stress in animal and pre-clinical studies is quite commonly extensively investigated and gives highly promising results. In general, experimental studies are focused on three different approaches to targeting oxidative stress during heart diseases: 1) inhibition of ROS generators, 2) enhancement of the endogenous antioxidant capacity, and 3) enhancement of the antioxidant capacity through supplementation with exogenous antioxidants (Fang et al. 2002).

In addition, early experimental studies show that an increase in endogenous antioxidant capacity leads to improved cardiac function in damaged heart models of rats and mice. These studies are particularly focused on primary antioxidant enzymes (SOD, CAT, and GPx) and on non-enzymatic antioxidants (NAD^+, GSH, α-tocopherol, and folic acid) (Fang et al. 2002).

Modification of the GSH status

Based on the positive results achieved by increased endogenous expression of the antioxidant enzymes, numerous experimental studies have focused on improving the endogenous antioxidant capacity.

The availability of cysteine intracellularly is a limiting factor in the synthesis of GSH. The administration of cysteine is problematic for both practical and safety reasons (Sochman and Peregrin 1992; Bourraindeloup et al. 2004; Ates et al. 2008). Cysteine oxidizes rapidly into insoluble cystine, making its production difficult. Additionally, cysteine is not resorbed fast enough by the cells, while extracellularly excessive cysteine is toxic and leads to neural degeneration and brain atrophy in mice (Kahns and Bundgaard 1990).

Cysteine involved in the production of GSH can be delivered *via* *N*-acetyl cysteine (NAC), ɤ-glutamylcysteine, and *L*-2-oxothiazolidine-4-carboxylic acid (OTC). In such a way, delivered cysteine will be used in the γ-glutamyl cycle for *de novo* synthesis of GSH (Figure 8) (van der Werf et al. 1971; Williamson et al. 1982).

The increase in endogenous antioxidant capacity is achieved primarily through precursor supplementation of the major cellular antioxidants GSH and NAD^+. The effectiveness and safety of this approach were demonstrated by the supplementation of NAC (precursor for GSH) in patients with heart damage and MI, and resulted in a better outcome for these patients (Williamson et al. 1982). Early studies focused on endogenous GSH enhancement by the administration of NAC showed that NAC can improve GSH levels, decrease oxidative stress, and improve cardiac function in rat models with myocardial injury (Bourraindeloup et al. 2004). NAC contains free SH groups capable of stimulating GSH synthesis, promoting detoxification, and acting directly as ROS 'cleaners' (Ates et al. 2008). Its use is limited due to the low membrane penetrability and low systemic bioavailability (Ates et al. 2008).

In addition to NAC, another GSH precursor, OTC (*L*-2-oxothiazolidine 4-carboxylic acid), known as pro-cysteine, was described and patented as a GSH delivery and enhancement

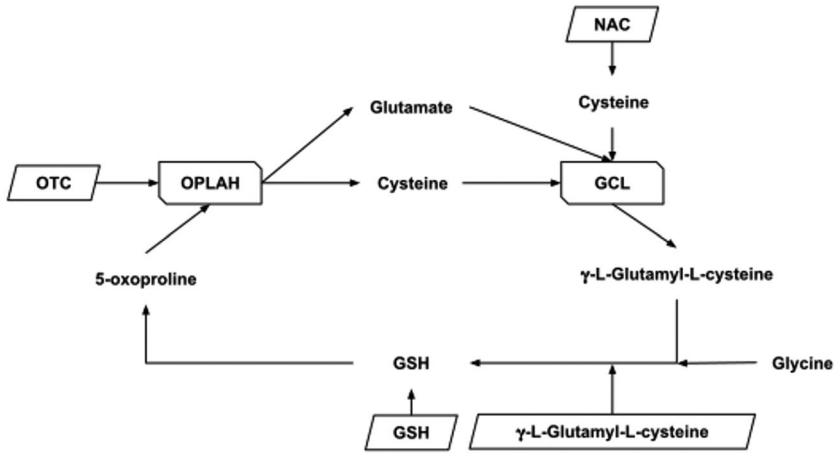

Figure 10. GSH synthesis from *N*-acetylcysteine, γ-glutamylcysteine, and *L*-2-oxothiazolidine-4-carboxylate (OTC) in the γ-glutamyl cycle. OTC is converted to cysteine, with 5-oxoprolinase (OPLAH) activity for *de novo* GSH synthesis. Similarly, NAC is converted intracellularly to cysteine and used to synthesize GSH. *L*-glutamylcysteine is used in the γ-glutamyl cycle to generate GSH by glycine addition. GCL: glutamate-cysteine ligase (modified from van der Pol et al. (2019)).

system (Williamson et al. 1982). OTC is effective in GSH synthesis because it is metabolized intracellularly to cysteine (Williamson and Meister 1982). 5-oxoprolinase, an enzyme found in many plant and animal cells, catalyzes the intracellular conversion of OTC to *L*-cysteine and CO_2 (Figure 10) (Meister et al. 1986). This compound exhibits a higher affinity for the enzyme than the natural substrate. Hence, 5-oxoprolinase converts OTC into *L*-cysteine, probably through the formation of the unstable intermediate compound S-carboxyl-*L*-cysteine (Figure 10) (Goldberg et al. 1992; Leaf and Pace 1994).

As mentioned before, feedback inhibition of γ-glutamylcysteine synthetase limits the excessive production of GSH, so that production of GSH in large quantities is not possible, despite the administration of high doses of OTC. Assuming that the sulfur atom of OTC is in the ring of the molecule, OTC does not undergo rapid oxidation and therefore is a stable cysteine/GSH precursor. Additionally, there are no deficiencies in the administration of OTC related to those in the administration of cysteine, such as high extracellular toxicity and limited cellular uptake (Goldberg et al. 1992). A study with radioactively labeled OTC (carbon 4 and 5, and carbon from the carboxyl group) showed that it is rapidly distributed and eliminated from the body (Zarka and Bridge 2017). The average half-life of OTC in the blood is approximately 1 h. Additionally, about 20–30% of the radioactive dose was exhaled as carbon dioxide in 24 h, indicating that OTC is metabolized throughout the Krebs cycle (TCA – tricarboxylic acid cycle). The incorporation of [^{35}S]-OTC into GSH was detected in all organs, but primarily in the kidneys and liver. [^{35}S]-taurine and cysteic acid have also been detected in the urine, which reflects the rapidity of cysteine metabolism (Weitberg 1987). Both γ-glutamyl-cysteine and OTC cause an increase in GSH levels and reduced oxidative stress in experimental and clinical studies (Zarka and Bridge 2017). OTC that is converted to cysteine by 5-oxoprolinase induces GSH to increase in experimental conditions (Meister et al. 1986). Interestingly, early experimental studies showed that supplementation with OTC induce improved cardiac function after myocardial injury (Shug and Madsen 1994). The effectiveness of the OTC treatment has been demonstrated in numerous large-scale animal studies, in research fields including cancer, lung and kidney damage, viral infections (HIV), and myocardial damage, but there is no data yet elucidating the *in vivo* OTC effect on MI.

Thanks to recent investigations, key information is obtained about the cell processes that underlie myocardial dysfunction associated with MI, which may be used as a basis for the development of future therapeutic interventions. In this direction, additional investigations based on the studies of the OTC's reparatory effects are on track.

Computational model associated predictive changes in the extent of oxygenation of the infarcted heart

The need to address the potential benefit of cross-examination of the biochemical (OTC-induced) positive effects after MI and computational models for the study of MI inevitably arise. Considering the role of OTC in the circulatory redox transformations described above, the exploitation of computational models for the study of oxygen transport from the capillary network in both infarcted and normal areas of the myocardium is imposed. These models can be used to reliably simulate the distribution of oxygen in infarcted rat hearts 1–4 weeks after MI and to simulate changes induced by co-interventions with OTC. Although most of the applied mathematical models are not intended to direct clinical treatment (drug dose, etc.), and to predict potential outcomes, the long-term goal of the computational approach is to develop a model that will be used in the prediction of clinical results after various interventions; whereby the consensus is that such a model may be feasible, especially based on the recent developments of the new noninvasive methodologies for the clinical measurement of the levels of hypoxia (Wang et al. 2007).

In general, the transport of oxygen to the tissue depends on the microcirculatory structure and its associated hemodynamics (Goldman and Popel 2000; Secomb et al. 2000; Beard and Bassingthwaighte 2001; Popel et al. 2003; Ziemer et al. 2003; Kaazempur-Mofrad et al. 2005; Wang et al. 2007). In order to develop an anatomically realistic computational

model of oxygen transport in the tissue, it is necessary to develop a detailed representation of the three-dimensional microscopic structures of the tissue vasculature (Beard 2001) and to describe the effects of the cellular structures (such as cell membrane and organelles) on the level of oxygenation (Ziemer et al. 2003). The accuracy of the modeling is fully dependent on the possibility to determine the rate of oxygen consumption at different levels of hypoxia, in different areas of the heart. Thereby, one of the basic limitations could be that certain models do not include all structural and functional components of the tissue (i.e. heterogeneous distribution of mitochondria in cardiomyocytes). The last is particularly important, especially for molecules like OTC (with well-examined intracellular and mitochondria-associated redox characteristics). However, the increasing availability of experimental data about the oxygenation status of post-infarcted tissue in dependence of the expected (OTC-induced) transformations, we believe, could result in an upgraded computational model that can be confirmed experimentally, and will be more useful for the future predictively associated cases.

This work untangled a portion of the observed animal models diversity, with the intention of bringing additional insight into disease knowledge, and greater translational success for innovative cardiac therapies. Understanding and predicting the 'critical points' in animal MI models can help to adapt studies and make realistic power calculations based on estimated damage, mortality, effective modifications, and possible experimental impact (Zwetsloot et al. 2017). This might lead to more precisely powered experiments, more definitive solutions to research problems, and less waste of experimental animals and research funds.

Conclusion

The basis of this manuscript is related to the perception that due to the increased antioxidant capacity, cardiac tissue will be able to overcome MI-associated changes and possibly prevent/attenuate the occurrence of oxidative stress, which is highly ranked among the factors responsible for the occurrence of acute MI.

The primary goal of future investigations will be to conduct a study focused on the effects of OTC treatment as a precursor responsible for the enhancement of the GSH-related antioxidative system capacity, and a molecule with anti-inflammatory properties as well. It is assumed that this will establish the basis for elucidation of the mechanisms through which applicable doses of OTC will manifest a potentially positive effect in the reduction of adverse effects of acute MI.

To evaluate the effect of OTC treatment, a future investigation will have to be done by determining the following: 1) Plasma Troponin I (cTnI) concentration as a specific marker of impaired myocardium during acute MI; 2) The concentration of markers for lipid peroxidation (MDA), oxidative protein damage (AOPP), and the concentration of total SH groups in the blood plasma and in the heart; 3) Changes in antioxidant status by determining the activity of antioxidant enzymes in the blood plasma and in the heart: CAT, SOD, GPx, and GR; 4) The state of antioxidant GSH status by determining blood plasma and heart GSH concentrations; 5) Histological analysis of the heart that will allow: detection of leukocyte infiltrations (inflammatory condition) and detection of necrotic tissue lesions; and 6) The transcriptional activity of NF-κB, most commonly initiated by pro-inflammatory processes.

The inclusion of OTC in the models for prediction of the distribution of oxygen in infarcted animal hearts can help to upgrade existing computational models. Such a model would be based on computational geometries of the heart, but the inclusion of biochemical redox features in addition to angiogenic therapy, despite improvement of the post-infarcted oxygenated outcome could enhance the accuracy of the oxygenation prediction.

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