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Study of molecular mechanisms in cardio- and neuroprotection and possibility of modulation by nutraceutical phytocomponents.

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CHAPTER 1

1. OXIDATIVE STRESS

1.1 *Definition*

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermedies or to repair the resulting damage.

Living organisms carry out their vital functions in an oxygen-rich environment and the majority of them suits and lives thanks to this. In a paradoxical way, oxygen should cause damage to important biological macromolecules of great importance. In biological systems a series of reactions that require energy occurs and cells use oxygen as an electron acceptor to produce energy. During these reactions , in physiological conditions, the production of free radicals occurs. The reason of the production of free radicals can be understood by studying how chemical reactions occur and the particular structure of molecular oxygen, which is essential for our metabolism. The production of free radicals is useful for the functioning of the cells, subcellular organelles, enzymes and for the synthesis and metabolism

of biomolecules. When they are not opposed by the organic defenses that their reactive potential becomes harmful.

Molecular oxygen is a di-radical with two unpaired electrons in its outer orbitals. These electrons are at their lowest level of energy and occupy separate orbitals, but with spin oriented in the same direction. Each of these orbitals can accommodate another electron, but the acquisition of electrons by another molecule and the formation of a bond is prevented by the fact that the spins are parallel (spin-restriction). Therefore, the spin reversal appears to be necessary for the formation of a bond. Molecular oxygen because of its status of di-radical and the phenomenon of spin-restriction, can not react with other free radicals. Chemically, a radical is a molecule containing a single unpaired electron in the orbital outermost, therefore tends to react with molecules of the surrounding environment and to which, by coming in contact, can subtract the electron needed to complete its orbital and achieve a steady state. The spin-restriction can be overcome with the assistance of catalyst such as, for example, the transition metals ions (iron and copper) that transfer an electron to time with the correct spin from the molecule that is to be oxidized to the oxygen that is reduced. Redox reactions will generate free radicals, such as superoxide anion or hydroxyl radical, but also other molecular species that are not free radicals but possess a similar reactivity as hydrogen peroxide and singlet oxygen. The term “reactive oxygen species (ROS)“ was coined to indicate all radicals with this reactivity. ROS are naturally formed because of normal oxygen metabolism. However these free radicals are potentially able to create oxidative

damage via-interaction with biomolecules. Obviously, ROS are not only always bad for normal physiology but sometime useful. For instance, lower amounts of ROS produced during mitochondrial activity in normal cells acts as the signaling molecules. The level of antioxidants and normal biological antioxidants must be in balance. If the mentioned balance is interrupted, then toxic oxidative stress may happen. This imbalance usually happens during aging (as an axample) or it can be involved in some diseases and also appears as a consequence of the diseases. The role of ROS in cellar damage is well documented with implications in a broad range of degenerative alterations (tissue degradation, carcinogenesis, ageing and other oxidative stress relate diseases).

1.2 Reactive oxygen species (ROS)

1.2.1 Superoxide anion

The superoxide anion is considered a free radical because it has an unpaired electron, although it not presents a particularly high reactivity. It is formed through the univalent reaction of a single electron. The superoxide radical can be formed at the level of two precise points of the mitochondrial respiratory chain: at the level of Complex I (NADH dehydrogenase) and Complex III (ubiquinone: cytochrome C reductase). In normal metabolic conditions, the Complex III is the main site of ROS production (Turrens 1997). In physiological conditions, high concentration of manganese superoxide dismutase enzyme in the matrix ensures that the basal level of

superoxide is neutralized before it can damage the cell. The overall redox balance of the cell turns out to be decisive as to what will happen to superoxide anion; in biological tissues, in non pathological conditions, it undergoes dismutation by superoxide dismutase (SOD) to generate O_2, according to the reaction:

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad [1]$$

1.2.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a reactive oxygen species and is produced by SOD depend dismutation reaction or is generated for direct production in some enzymatic reactions, as those catalyzed by L-amino acid oxidase or monoamine oxidase at the level of microsomes, peroxisomes and mitochondria. In physiological conditions, also H_2O_2 does not represent a particular risk for the cell, since it is rapidly neutralized by the action of catalase, a ubiquitary heme protein and glutathione peroxidase according to the reactions:

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O \qquad [2]$$

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG \qquad [3]$$

Even though hydrogen peroxide can control cell signaling and stimulates cell proliferation at low levels, in higher concentrations it can initiate apoptosis in very hight levels it may create necrosis (Abdollahi et al. 2012; Saeidnia et al. 2013).

1.2.3 Hydroxyl radical

The free radical produced in higher quantities is the superoxide anion. It may react with H_2O_2 to form the dangerous and powerful hydroxyl radical $OH^{\bullet}$ (Haber-Weiss's reaction):

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^- \quad [4]$$

The hydroxyl radical is formed especially in the redox reaction of H_2O_2 with iron or other transition metals according with Fenton reaction [5].

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad [5]$$

Fe^{3+} so formed may, in turn, interact with a superoxide radical according to the reaction [6]:

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2 \quad [6]$$

regenerating Fe^{2+} which can again react according to [5] with a new molecule of H_2O_2.

The production of $OH^{\bullet}$ occurs only in the presence of free iron. Iron, at physiological pH, in plasma and tissues, is always present in bound , non-toxic form (Fe-hemoglobin, Fe-myoglobin, Fe-citochrome etc.)

but just a slight shift in pH toward acidosis promotes the separation of the metal from the protein and the initiation of radical reactions.

The hydroxyl radical OH• is the agent responsible for the initial phase of many peroxidative processes in our tissues. Actually, in the presence of transition metals, hydrogen peroxide can be converted to the hightly reactive idroxyl radical, which is responsible for most of the oxidative damages to proteins, lipids, sugars and nucleic acids. Hydroxyl radical is also a a hallmark signaling molecule that is able to inactivate NF-kB, an important transcription factor involved in infiammatory response.

1.2.4 Singlet oxygen

A highly reactive form of molecular oxygen is singlet oxygen ($^1O_2$). It has the ability to oxidize the organic molecules damaging living systems. In vitro studies it showed that $^1O_2$ oxidizes many organic molecules, including membrane lipids, proteins, amino acids, nucleic acids, nucleotides, carbohydrates and thiols. Singlet oxygen can be formed during the action of phagocytes when they are acting as agents of non-specific immunity. It has been hypothesized that $^1O_2$ is a by-product of lipid peroxidation and recent studies have shown that it can also be formed as the primary product of Haber-Weiss's reaction [7]

$$O_2^{-\bullet} + H_2O_2 \longrightarrow {}^1O_2 + OH^\bullet + OH^- \qquad [7]$$

Mitochondrial respiration generates a proton gradient and singlet oxygen perhaps as a signaling element that might be involved in oxidative stress and alkaline-induced cell death (Mates et al. 2012).

The creation of $O_2^{-\bullet}$ following irradiation seems to be a main character of cell injury (Tominaga et al. 2012). Since manganese superoxide dismutase (Mn-SOD) removes extra $O_2^{-\bullet}$ in the mitochondria to preserv them from oxidative damage, thus over-espression of Mn-SOD reduce the levels of intracellular ROS and protects against cell death.

1.3 Endogenous ROS formation

The transformation of molecules in free radicals occurs through a chemical or physical reaction. In fact it is well known that many external agents such us UV rays and X-rays are able to generate free radicals within biological systems. ROS can be also generated by the cell at the level of the mitochondrial respiratory chain. Experimental tests in vitro indicate that mitochondria convert 1-2% of the molecules of oxygen consumed in superoxide anion. Molecular oxygen is the ideal final acceptor of electrons in the mitochondrial respiratory chain, presenting high affinity for electrons, combined with a low reactivity in the absence of a catalyst.

1.4 Reactive nitrogen species

Nitric oxide and peroxynitrite, which is formed by the reaction between nitric oxide and superoxide, represent the reactive nitrogen

species that have attracted most interest. Class of enzymes belonging to the family of nitric oxide synthetase or synthase (NOS), using L - arginine as a substrate, is assigned to the synthesis of nitric oxide (NO). The nitric oxide synthase are a family of NADPH-dependent enzymes that oxidize the amino acid L-arginine to L-citrulline. NOS can be found in three different forms: neuronal, endothelial and inducible (iNOS). While the first two are calcium/calmodulin-dependent, the iNOS is calcium independent and is expressed in response to different stimuli. The cell has the ability to produce large amounts of nitric oxide as it is able to regulate the expression of iNOS. NO exists in different chemical forms, with high chemical reactivity and has different biological roles. NO is produced by epithelial cells and this amount, if remains at physiological levels, appears to be essential for the proliferation, regolation and relaxation of vascular smooth muscle cells, for leukocyte adhesion and aggregation of platelets (Knowles et al. 1992). NO has different functions according to the produced levels: at low levels, produced by neuronal constitutive NOS, serves as a neurotransmitter (Moncada et al. 1991), while produced at high levels by activated macrophages and neutrophils it is an important mediator of the immune response (Bogdan 2001).

1.6 ROS-producing enzymes contributing to oxidative stress

Several enzymes systems that can produce ROS have been identified. Four seem to be of major importance: namely, NADPH oxidase,

xanthine oxidase, enzymes of the mitochondrial respiratory chain and dysfunctional eNOS.

1.6.1 NADPH oxidases

NADPH oxidase produces $O_2^{-\bullet}$ by transferring electrons from NADPH inside the cell across the cell membrane and reducing molecular oxygen. Several isoform of NADPH oxidase (Nox) are found in various cell types of the vascular wall, including endothelial cells, smooth muscle cells and fibroblasts (Drummond et al. 2011). NADPH oxidase comprises multiple protein components that must be assembled in the cell membrane for the enzymes to become active(Drummond et al. 2011). In various animal model vascular disease, activation and/or upregulation of NADPH oxidase has been demonstrated in the vessel wall. Mice with a disrupted Nox1 gene exhibit smaller blood pressure increases in response to angiotensin II (Matsuno et al. 2005), whereas mice overespressing Nox1 in vascular smooth muscle exhibit increased $O_2^{-\bullet}$ production and greater blood pressure in response to angiotensin II (Dikalova et al. 2005). Disruption of the gene for the regulatory subunit p47phox reduces blood pressure responses to angiotensin II and diminishes atherogenesis in apolipoprotein E (ApoE) $^{-/-}$ mice (Barry-Lane et al. 2001).

1.6.2 Xanthine oxidase (XO)

The main source of XO is the liver. It primarly synthesizes xanthine dehydrogenase, which is then converted to XO by proteolysis. The

release of XO from the liver is increased in hypercholesterolemia (White et al. 1996). The circulating XO can then adhere to endothelial cells by assiociating with endothelial glycosaminoglycans. XO donates electrons to molecular oxygen, thereby producing $O_2^{-\bullet}$ and hydrogen peroxide. Allopurinol and oxypurinol are inhibitors of XO. Oxypurinol has been shown to reduce $O_2^{-\bullet}$ production and improve endothelial function in blood vessels from hyperlipidemic animals (Ohara et al. 1993). This suggest a contribution of XO to endothelial ROS production, at least in hypercholesterolemia. There is evidence that endothelial cells themselves can express xanthine dehydrogenase and that this expression is upregulated in a redox-sensitive way when the activity of endothelial NADPH oxidase in increased.

1.6.3 Enzymes of mitochondrial respiratory chain

Mitochodria produce substantial amounts of $O_2^{-\bullet}$ in ETC complex I and III. Complex I releases $O_2^{-\bullet}$ into the mitochondrial matrix and is considered the main producer of $O_2^{-\bullet}$ due to reverse electron flow from complex II under low-ADP conditions. The matrix-localized mitochondrial SOD2 dismutates $O_2^{-\bullet}$ to H_2O_2, which in turn is reduced to water by GPx or catalse. The importance of SOD2 thus lies in the detoxification of $O_2^{-\bullet}$ to prevent generation of $ONOO^-$ or oxidative damage of ETC proteins and mitochondrial DNA, which may otherways compromise mitochondrial function. Complex III also releases $O_2^{-\bullet}$ to the mitochondrial intermembrane space, where it is dismutated by SOD1, Mitochondrial ROS (Mito-ROS) production varies depending on several factors. Surprisingly, mitochondria

themselves are remarkably ROS sensitive. Oxidative damage lowers their activity and increases their ROS production (Davidson et al. 2007). Mito-ROS promote the activity of other ROS sources (Schulz et al. 2012). Enhanced levels of mito-ROS are known to stimulate the release of mitochondrial apoptotic factors, with vascular cells showing differential sensitivity to stress conditions (Zeini et al. 2007). In the atherosclerotic process, an increase in cell death contributes to the formation of a necrotic core in plaque, which renders it unstable, favoring atherothrombosis (Madamanchi et al. 2007). This hightlights the role of oxidative stress in vascular diseases, a phenomen exemplified by the fact that elevated mito-ROS levels cause cardiomyopathy in $SOD2^{-/-}$ mice (Li et al. 1995).

1.6.4 Dysfuntional uncoupled eNOS

A key regulator of the vasoprotective function of the endothelium is generated by eNOS. Endothelial NO induces vasodilation, inhibits platelet aggregation and adhesion, and prevent atherogenesis (Forstermann 2008; Forstermann et al. 2011). Endothelial dysfunction is largely equivalent to the inability of the endothelium to generate adequate amounts of bioactive NO. Oxidative stress contributes markedly to endothelial dysfunction, primarily due to rapid oxidative inactivation of NO by excess $O_2^{-\bullet}$. In a second step, persisting oxidative stress renders eNOS dysfunctional, such that it no longer produces NO, but rather $O_2^{-\bullet}$. Evidence for eNOS uncoupling has been obtained in various animal models and in patients with endothelial dysfunction (Forstermann et al. 2011). Mechanistically,

depletion of BH_4, an essential cofactor for the eNOS enzyme, is likely to be the major cause for eNOS uncoupling and endothelial dysfunction. $ONOO^-$, the direct reaction product of NO and $O_2^{-\bullet}$, is capable of oxidizing BH_4, leading to BH_4 deficiency (Forstermann 2008)

1.7 Relationship between oxidative stress and cardiovascular diseases

Oxidative stress plays a a central role in cardiovascular diseases (Li 2013, Salari-abdollahi 2012). There are some risk factors of cardiovascular diseases including hypertension , hypercholesteremia, diabetes mellitus and cigarette smoking, as well as cardiovascular diseases that itself causes significant augmentation of ROS in the vascular wall, a situation that eventually culminates with oxidative stress. In the state of oxidative stress, enzymatic production of ROS exceeds the avaible antioxidant defence system. The ROS produced include free oxygen radicals, oxygen ions, and peroxides. Superoxide reacts with nitric oxide and lead to loss NO bioactivity. The resulting peroxynitrite may cause dysfunction of eNOS and thus reduce NO production. The reduced bioavailability of vascular NO is the major mechanism of endothelial dysfunction observed in cardiovascular diseases. In addition, vascular oxidative stress promotes atherogenesis througt numerous different mechanisms including the activation of redox-sensitive transcription factors and induction of lipid

peroxidation, protein oxidation, and mitochondrial and nuclear DNA damage (Forsterman 2010).

1.7.1 Oxidative stress and hypertension

Numerous studies in hypertensive patients have shown high levels of free radicals (Lacy et al. 1998). Endothelial cells are critical in combating hypertension as they play a key role in arterial relaxation. No is one of the agents that with its vasodilatory action, helps to fight hypertension (Vallance 1998). In the presence of superoxide anion, NO has a very short half life as it is degraded by it. Therefore, the superoxide anion has the ability to modify endothelial function being a major determinant of nitric oxide (NO) biosynthesis and bioavailability. It acts therefore as a vasoconstrictor. eNOS can generate superoxide rather than NO in response to a variety of stimuli. As a result, eNOS may become a peroxynitrite generator, leading to a dramatic increase in oxidative stress, since peroxynitrite formed by the NO-superoxide reaction has additional detrimental effects on vascular function. So oxidative stress is evident in different types of hypertension, including angiotensin II-induced hypertension, spontaneously hypertensive rats (SHrs) – tha animal model of genetic hypertension, and deoxycorticosterone acetate (DOCA)-salt hypertension, which is a low –renin/angiotensin hypertension model. In all three types of hypertension NADPH oxidase is likely to represent the major and primary ROS source. Genetic deletion or inibhition of NADPH oxidase lowers blood pressure in hypertension models (Matsuno 2005, Landmesser 2003, Chalupski 2005). eNos

encoupling has been documented in all three and contributes significantly to vascular oxidative stress. eNOS encoupling in hypertension seems to be secondary to NADPH oxidase activation and mainly attributable to NADPH oxidase –mediated BH_4 oxidation (Landmesser 2003). In angiotensin II-induced eNOS encoupling, BH_4, deficiency is additionally caused by reduced BH_4, recycling from BH_2 due to downregolation of endothelial dihydrofolate reductase (Chalupski 2005).

1.7.2 Oxitadive stress and diabetes

Studies on animals and men suggest that oxidative stress plays an important role in the pathogenesis of both types of diabetes mellitus (Maritim et al. 2003). The increased production of ROS seems to be the main cause of the pro-inflammatory state and endothelial dysfunction which stands at the origin of vascular injury. Hyperglycemia stimulates cellular ROS production by different sources, with the mitochondrial ETC acting as the initial $O_2^{-\bullet}$ producer (Giacco et al. 2010). Overproduction of mitochondria derived $O_2^{-\bullet}$ leads to activation of protein kinase C (PKC) and formation of advanced glycation end-products (AGEs). PKC and AGEs can, on the hand, activate NADPH oxidase and, on the other hand, inhibit eNOS activity through post-translational modifications (Funk et al. 2012). Uncoupling of eNOS has been demonstrated in streptozotocin induced type diabetes (Hink et al. 2001). BH_4 oxidation and BH_4 deficiency due to oxidative stress is likely to be a major cause of eNOS uncoupling under these conditions. Insulin resistance in type 2

diabetes may decrease GTP cyclohydrolase 1 activity and thus reduce BH_4 de novo synthesis. The increased levels of angiotensin II in patients with diabetes may additionally reduce DHFR expression and thus BH_4 recycling from BH_2 (Chalupsky et al. 2005).

1.7.3 Oxidative stress and atherosclerosis

Atherosclerosis involves LDL entrapment in the arterial wall and an inflammatory response to the local LDL. The complexity of this process precludes a thorough discussion here. Nevertheless, common features of atherosclerosis include LDL oxidation, endothelial dysfunction, and inflammation (Stocker et al. 2004). Importantly, these 3 features of atherosclerosis all involve ROS in their pathophysiology. The oxidation of LDL is a well-described phenomenon in atherosclerosis. A number of enzyme systems have been proposed to contribute to LDL oxidation in vivo, and there are a number of reviews available on this topic (Levitan et al.). The most complete data concerning ROS-mediated LDL oxidation in vivo involve the contributions of NADPH oxidases and mitochondria. Human atherosclerotic coronary arteries contain increased immunostaining of p22phox (Azumi et al. 1999), an NADPH oxidase subunit. This protein is principally associated with Nox2 in lesional macrophages, and the p22phox expression level is positively associated with atherosclerosis severity (Sorescu et al. 2002). Animal studies using the ApoE-null atherosclerosis model indicate that mice lacking the Nox2 isoform of NADPH oxidase exhibit a 50 %

reduction in lesions, along with a marked decrease in aortic ROS production, suggesting that inhibition of Nox2 NAPDPH oxidase could limit atherosclerosis(Judkins et al. 2010). Humans express an NADPH oxidase isoform (Nox5) that is not found in rodents, and this oxidase may also contribute to blood vessel ROS as coronary arteries with atherosclerosis exhibit an increased expression level and activity of Nox5 (Guzik et al. 2008). Mitochondrial ROS have been implicated in many chronic diseases, including atherosclerosis (Corral et al. 1992). Emerging data now link mitochondrial ROS production to the control of inflammation. For example, mitochondrial ROS are important for signaling events critical to innate immunity(West et al.) and activation of the NLRP3 inflammasome (Zhou et al. 2011) that is known to contribute to both animal and human atherosclerosis (Duewell et al.). As atherosclerotic lesions mature, they develop a fibrous cap overlying a lipid core. Acute vascular events are often caused by weakening of this fibrous cap and plaque necrosis is a key mechanism for fibrous cap weakening and rupture (Virmani et al. 2000). The apoptosis of macrophages and the inability to clear these apoptotic cells are key contributors to plaque necrosis. When macrophages become apoptotic, they also stimulate a process known as autophagy in which the cells consume their own cellular components in an organized manner. It turns out that autophagy is an important adaptive mechanism for oxidative stress and without autophagy, ROS production (via Nox2 NADPH oxidase) is increased, and plaques become more prone to rupture. Thus, oxidative stress is

dependent upon autophagy, and recent data suggests that stimulating autophagy could have beneficial effects for atherosclerosis.

1.7.4 Oxidative stress and myocardial injury

ROS are the main cause of myocardial injury during ischemia/reperfusion (Duilio et al. 2001). In numerous studies conducted on infarcted animal models it was observed that the levels of ROS are very high (Cargnoni et al. 2000). It has been reported that the development of myocardial ischaemia/reperfusion injury is associated with an increase in the population of apoptotic cells in the peri-necrotic area (Zhao et al. 2002). Neutrophils are the primary source of ROS during reperfusion. Also endothelial cells and cardiomyocytes can generate ROS. ROS are produced from xanthine oxidase in endothelial cells, mitochondrial electron transport chain reactions in cardiomyocytes, and NADPH oxidase in inflammatory cells (Waypa et al. 2002). Even in the correlation between oxidative stress and myocardial injury a key role is attributed to the action of NADPH oxidase. In fact, it has been observed that the activity of this enzyme is increased in the tissue of the left ventricle in animals with heart failure (Li et al. 2002). In studies in rats suffering from myocardial infarction an increase in the production of ROS was observed, with a decrease in the formation and bioavailability of endothelial NO (Wiemer et al. 2001).

1.7.5 Oxitadive stress and acute ischemia

In recent decades myocardial ischemia is the leading cause of death worldwide. The major cause is the reduction of blood influx to the heart due to the presence of atheromatous plaques in the arteries. In order to limite the damage due to this phenomenon, the reperfusion should be performed immediately after the ischemic insult, although reperfusion occuring after itself would be responsible for the formation of ROS (Bolli 1992). The increase in intracellular production of ROS and therefore the increase of oxidative stress is not the only phenomenon that is found in the hearts subjected to damage of myocardial ischemia/reperfusion injury, but in addition cardiac dysfunction, reduction of antioxidant potential, increase of lipid peroxidation are present. It has been observed that exposing the heart or the sub-cellular organelles to oxidative stress, the same deleterious effects observed in hearts affected by I/R are detected (Siveski-Iliskovic et al. 1995; Dhalla et al. 1996). One aspect that can not be omitted is the fact that a small amount of ROS is produced during mitochondrial respiration; instead, during ischemia, the mitochondrial transporters are in a reduced state (Freeman et al. 1982). An increased production of superoxide anion due to increase in the loss of electrons by the respiratory chain is observed. In reperfusion there is a further loss of electrons which causes an increase in the production of superoxide anion (Schmid-Schonbein 1982).

1.7.6 Oxidative stress and smoke

Compounds contained in cigarette smoke have been shown to activate endothelial NADPH oxidase (Jaimes et al. 2004) and impair mitochondrial function, threby elicitng mitochondrial oxidative stress (Csiszar et al. 2009). The increased $O_2^{-\bullet}$ and $ONOO^-$ production induces vascular inflammation and DNA damage and accelerates vascular ageing (Csiszar et al. 2009). Moreover, compounds from cigarette smoke may potentiate the pro-oxidative activity of LDL by inducing oxidative modifications of LDL (Steffen et al. 2012). The endothelial dysfunction seen in smokers is largely attributable to BH_4 deficiency due to oxidative degradation. Supplementation by BH_4 can correct endothelial dysfunction in smokers (Heitzer et al. 2000).

1.7.7 Oxidative stress and physical activity

Physical activity increases the generation of free radicals in several ways. Two to 5% of oxygen used in the mitochondria forms free radicals. As oxidative phosphorylation increases in response to exercise, there will be a concomitant increase in free radicals. Catecholamines that are released during exercise can lead to free radical production. Other sources of free radical increase with exercise are xanthine oxidase, NAD(P)H oxidase, and several secondary sources, such as the release of radicals by macrophages recruited to repair damaged tissue. This increase, even if is required to meet the energy requirements on the other side leads to an increased production of free radicals. The amount of free radicals produced is directly proportional to the duration and intensity of exercise and inversely to

the degree of trainingof those who practice it. In fact, training improves the antioxidant capacity of the organism and allows trained athletes to more efficiently counteract the produced free radicals. Conversely, the greater risk may be that, in an untrained individual, high intensity physical exercise can lead to the production of free radicals not counteracted by the antioxidant defenses of the organism.

CHAPTER 2

2. Ischemic preconditioning

2.1. Ischemia and reperfusion of the myocardium

Myocardial ischemia is the leading cause of death in the modern world. The main risk factors responsible for the onset of this disease are hypertension, ventricular hypertrophy, hyperlipidemia, atherosclerosis, diabetes, heart failure and aging. These factors can affect the development of ischemia and interfere with responses to cardioprotective interventions. A significant improve in the treatment of acute ischemic heart disease can be obtained by using procedures that enable the rapid restoration of blood flow in the ischemic area of the myocardium affected by the insult. These procedures are called of therapy of reperfusion. Although reperfusion is essential for the survival of the organ,it is, especially in the early stages, the cause of an aggravation of the injuries caused by the initial ischemic insult. The damageat the cardiac level induced by an ischemic event are not

solely due to ischemia, but also reperfusion (I/R damage). The complications of reperfusion include myocardial stunning and arrhythmias. The term stunning refers to a loss of contractility immediately following a sub-lethal ischemic insult. If the reperfusion and revascularization of the infarct zone are carried out promptly, myocardial contractility recover completely even though the recovery may take several hours or days. Contrary to an infarcted heart, the stunned myocardium recovers fully its activities. The identification of cardioprotective agents is very important to improve myocardial function and reduce the incidence of arrhythmias, delay necrosis and limit infarct size.

In recent years, several studies have shown that the heart is capable of triggering mechanisms of adaptation to counteract the damage caused by I /R. These mechanisms are the main target of scientific research.

Ischemia of short duration (2–10 min) protects the heart against damage that may arise with a next ischemic insult. This important phenomenon, known as ischemic preconditioning (PC), is an adaptive response, in which exposure to a brief I/R significantly increases the heart's ability to support a next sequence of ischemic injury. Unfortunately, the limitation of this mechanism is that it has a limited effect in time and in most cases ischemic attacks can not be predicted. This limitation is not present in the high-risk angioplasty where ischemic PC may have some practical utility. Recent studies conducted “in vivo” and in isolated hearts have demonstrated that we can achieve significant reductions in reperfusion damage, making a series of very brief re-thrombosis and coronary reperfusion at the

beginning of reperfusion. This phenomenon has been referred to by the term “ischemic postconditioning”.

2.2 Reperfusion injury

Irreversible ultrastructural changes occurr in the infarcted myocardium. If reperfusion of the tissue is carried out in a short time, changes may be considered reversible. Coronary occlusion lasting 20-30 minutes leads to a transition from a state of reversible changes to a state of tissue injury and necrosis of cardiac cells (Jennings et al. 1981). It has been shown that a diversified number of risk factors, influencing the onset of irreversible lesions such as the size of the affected area, the extent of collateral blood flow or the residual arterial flow, the ischemic duration (Miki et al. 1998).

Reperfusion is a fundamental process for the recovery, as in the absence of reperfusion, no intervention is able to limit the development of infarction. In acute myocardial infarction, revascularization and reperfusion are the main therapies that can save the organ which can be irreversibly damaged, limiting the extent of necrosis. Mortality for myocardial infarction, is linked to the duration of coronary occlusion (Bishopric et al. 2001). In the myocardium subjected to I /R, cell death can occur either by necrosis and by apoptosis; while the necrosis can be caused both by ischemia and reperfusion, apoptosis is induced mainly by reperfusion. The first experimental studies on apoptosis using permanent coronary occlusion in rats, have shown that apoptosis is the principal cause of death of myocytes. Subsequently, most of the experimental tests, have

suggested that the number of cells undergoing apoptosis is probably less than the number of necrotic cells. It is not yet clear how these two processes are linked. The restoration of blood flow in the coronary artery branch, perfusing the ischemic myocardium, leads to the production of superoxide anion by different enzyme complexes. The superoxide anion, along with other ROS, exerts a strong oxidizing action on the myocardial fibers already damaged by ischemia. This situation leads the cells to apoptosis. Furthermore, the superoxide anion formed can react with nitric oxide to formed peroxide nitrite ($NOOO^-$). The production of $NOOO^-$ reduces the bioavailability of NO but also participates with $O_2^{-\bullet}$ to tissue injury, being also equipped with oxidizing power. The deleterious action of $O_2^{-\bullet}$ is reduced due to its conversion to hydrogen peroxide by SOD. However, as in the presence of Fe^{2+} or Cu^{3+}, the hydrogen peroxide can be converted into hydroxyl anion $HO^-\bullet$, that is more reactive than superoxide anion and hydrogen peroxide. An apparent initial decrease may be followed by an increase in toxicity. The cellular calcium overload contributes to myocardial reperfusion injury. This phenomenon, which manifests itself under conditions of anoxia, is due to the malfunctioning of specific Ca^{2+} pumps, that are situated on the sarcolemma and on longitudinal tubules and sodium pump with consequent entry of Ca^{2+} and activation of the Na^+/Ca^{2+} exchanger. The Ca^{2+} overload also promotes the expression of pro-apoptotic genes. The nuclear factor NFkB plays a key role in reperfusion. NFkB accentuates the myocardial injury recalling inflammatory reactions. Its activation is induced by various agents, including hydrogen peroxide. Furthermore,

the reduced NO availability , induced by I/R, causes an up-regulation of cell adhesion molecules that promotes the adhesion of leukocytes to the endothelium and their eventual migration to the myocardial fibers. NF-kB is also involved in activation of gene transcription coding for cell adhesion molecules. The cellular Ca^{2+} overload, ROS production, NFkB activation and cell adhesion molecules, can lead to the phenomenon of "no-reflow". This phenomenon increases the damage of the myocardium induced by the preceding ischemic insult. Until a few years ago, ischemia was considered the cause of cell death that occurs basically due to the depletion of ATP. In these conditions the reperfusion appears to be essential to restore the synthesis of ATP, and thus to save the not damaged cells Reperfusion accelerates cell death of all those cells that are already irreparably damaged and sentenced to death. It is only in the last 10 years that some studies have identified the importance of mitochondria in this context. Mitochondria play a decisive role in determining cell fate during and after cellular stress (Kroemer et al. 2007). A fundamental component of the mitochondrial response to stress is the formation of 1- methyl-4-phenyl-1,2,3,6-tetrahydropyridine (mPTP), a multimeric structure ranging from the inner membrane to the outer membrane of the mitochondria (Halestrap et al. 2003). The only known component of the channel is cyclophilin D, an isomerase that binds to and is inhibited by cyclosporin A (Crompton et al. 1988). The mPTP are non selective voltage dependent channels which are closed in normal physiological condition, when the mitochondrial membrane is impermeable to most solutes. Under conditions of cellular stress, the

opening of pores in the inner membrane, leads to loss of impermeability of the membrane and the rapid collapse of mitochondrial potential. The factors that determine whether and when the mPTP open during I/R and the evidence that their openness can contribute to I/R, have been extensively studied over the past decades. The most recent hypothesis suggests that the opening of the pores does not occur during ischemia but during the early stages of reperfusion and that inhibition of the opening of the pores protects the cell from necrotic death. The formation or inhibition of mPTP during reperfusion is the primary determinant of cell death or survival. Some studies show that the opening of the mPTP leads to cell die by necrosis, while others indicate that, depending on the extension of the opening of the mPTP, death occurs by apoptosis or necrosis (Bernardi et al. 2006). The corollary of this new vision is that specific manipulations, during reperfusion offer the potential to attenuate cell death through cardioprotective strategies implemented during reperfusion. Therefore, PC, pharmacological treatments that mimic PC the postconditioning and selected agents administered during reperfusion may protect the cell by attenuating the opening and extension of mPTP during reperfusion. In conclusion, reperfusion is essential to save the infarcted organ. However, it can cause further irreversible damage. These damages are closely related to the previous ischemia that is related to the opening of mPTP in the first moments of reperfusion. Further researches are needed to evaluate the potential of reperfusion as a therapeutic intervention in order to maximize its benefits in acute myocardial infarction.

2.3 Preconditioning

Preconditioning is the phenomenon whereby brief episodes of ischemia and reperfusion protect the heart from ischemic injury due to a subsequent ischemic insult. Thus, ischemic PC is a protective and adaptive mechanism obtained by short periods of ischemic stress rendering the heart resistant against another similar or greater ischemic event. PC was described by (Murry et al. 1986). In this experiment, authors exposed a group of open-chest dogs to a four cycles of 5 min coronary occlusions followed by 5 min of reperfusion and then exposed them to a 40 min coronary occlusion followed by 4 day of reperfusion. Controls received only 40 min coronary occlusion., The result was that the dogs receiving 4 cyclic events of 5 min occlusion, followed by 5 min reperfusion before extended ischemic episode, had a much smaller myocardial infract size compared with control group. This cardioprotective effect of preconditioning was attributed to a rapid metabolic adaptation of the ischemic myocardium. Any studies have highlighted the transient nature of this phenomenon. It has been demonstrated in rabbit heart that protection against infarction, obtained by a single period of 5 min preconditioning , is ineffective if the interval between the PC stimulus and myocardial infarction protocol exceeds 60 min (Liu et al. 1991). In 1993, PC has been demonstrated to have a biphasic pattern (Kuzuya et al. 1993; Marber et al. 1993): an early phase, starting within 1h from preconditioning and ending after about 2h, and a late phase, developing after 24 hours of preconditioning and lasting up to 72 hours. Since 1990 numerous articles have been published on various

aspects of PC in the myocardium and other tissues; most of them turned their attention to the classical preconditioning.

2.4 Late preconditioning

The late phase develops 12 to 24h after the first PC stimulus and last 72 to 96h, although the importance of protection may be somewhat less than in the early phase . Unlike the early phase, the late PC phase has an effect against myocardial infarction but also against myocardial stunning (Bolli 1996). Because of its 30- to 50-fold longer duration and the broader protection it provides many studies have been focused on the late phase and its clinical exploitation (Bolli 2000). The late PC was initially considered an adaptive phenomenon mechanistically distinct from the classic PC. Merber et all in their early studies examined the hypothesis that a transient stress (Marber et al. 1993), caused by I/R, could lead to the de novo synthesis of a cytoprotective protein. This protein is the 72kDa heat shock protein or HSP72. They demonstrated a correlation between the increase of expression of this protein and the decrease of myocardial infarction damage, after 24 hours of ischemic preconditioning in rabbit hearts. A stress response is the induction of cytoprotective factors, such as cellular antioxidants and HSP. However, the factors influencing the induction of cytoprotective components, have not yet been well defined in mammals (Hoshida et al. 1993). The phenomenon of late PC can be induced by broad multiplicity of stimuli, which can be divided in non pharmacological and pharmacological. The first group includes exercise, hypoxia, ischemia, rapid ventricular pacing and heat

stress (Bolli 2000); the second consist of naturally occurring and often noxius agents, including endotoxin, ROS (Tang et al. 2005), tumor necrosis factor-α (TNF-α), TNF-β, leukemia inhibitory factor and of clinically applicable drugs including endotoxin derivates such as monophosphoryl lipid, adenosine receptor (AR) agonists (Takano et al. 2001), NO-releasing agents, opioid receptor agonists and α_1-adrenoceptor agonists. As mentioned above, late PC is the result of a complex cascade of signaling events that ultimately results in cardioprotection. Brief events of myocardial I/R, hypoxia, and heat stress are associated with metabolic perturbation that results in the generation of a wide variety of metabolites and ligands. Among these, adenosine, ROS, catecholamines, endogenous opioids and NO act as chemical signals that trigger the development of the late PC. These agents can induce pharmacological PC in the absence of ischemia. Among these, NO has a key role in late PC. NO, formed in the early PC, is essential to induce this cardioprotective effect. Another study demonstrated that NO is also necessary to trigger late PC against myocardial infarction . It was observed that late PC is also induced by exposure to exogenous NO, because the treatment with NO donors in the absence of ischemia induces a loss of protective effect against both myocardial stunning and infarction. Chemical signals trigger the development of late PC by inducing a series of complex signaling events. The signaling pathways involved in these mechanisms are protein kinase C (PKC), Src protein tyrosine kinases (Src PTKs), mitogen-activated protein kinases (MAPKs), JNKs, NFkB and signal transducers and activators of trascription (STATs). The general

pattern can be described starting from the interaction of NO derived from eNOS with the superoxide anion to form peroxynitrite, which activates PKC. This signaling pathway activates Src and Lck. The NFkB activation occurs thanks to the double phosphorylation of a serine and a tyrosine on the inhibitory protein IKB – α by PKC and tyrosine kinases. Cytoprotective proteins are induced, at the gene level, by NFkB. Among these are the inducible form of NOS (iNOS) and cyclooxygenase 2 (COX -2). NO produced by iNOS seems to regulate the activation of COX -2 in the preconditioned myocardium, inducing the generation of prostanoids, which is critical for the establishment of the cardioprotective phenotype (Bolli et al. 2002). The dependence of late PC by upregulation of iNOS and COX-2 is clearly demonstrated by several pharmacological and functional genomics studies, that involve pharmacological inhibition and genetic deletion of iNOS and COX-2 (Bolli et al. 2002). It is not yet known how prostanoids derived from COX-2 play their cytoprotective action and their relationships with other cytoprotective mechanisms, such as antioxidant enzymes (Ockaili et al. 2005), HSP, and inhibition of mPTP. Studies using microarrays have observed that PC is able to change the pattern of gene expression in rat heart. This suggests that complex cellular mechanisms are involved in the evolution of cardioprotection afforded by PC (Onody et al. 2003).

2.5 Postconditioning

The main limitation of ischemic PC is the not predictability of ischemic events except in special cases such as heart transplants or heart surgery. For this reason the identification of therapies that can be executed in the presence of heart infarction or in successive moments is of primary importance. The first studies on cardiac conditioning performed during the reperfusion were carried out for this aim. The results obtained led to the definition of a therapeutic treatment, ischemic postconditioning. In anesthetized dogs subjected to prolonged myocardial ischemia, three occlusions of 30 seconds each from 30 seconds after the start of reperfusion, led to a significant reduction of the infarct zone, tissue edema and post-ischemic endothelial dysfunction, compared with dogs that had not undergone coronary occlusions. This technique allowed to obtain a protective effect almost equal to that obtained with ischemic preconditioning. Postconditioning protocol reduces infarct extension only when performed in the early stage of reperfusion. Recently, the postconditioning has also been successfully applied in humans. In isolated rats hearts, cardioprotection by postconditioning is mediated by inhibition of ROS formation and the first few minutes of reperfusion are critical for cardioprotective effects, due to the peak in ROS production. It should however be noted that, depending on the animal species and the duration of ischemia, the time at which the peak of ROS production appears is different. In fact, while in the rabbit heart subjected to 10 minutes global ischemia, the maximum

release of ROS takes place 10 seconds after the start of reperfusion; in the hearts of dogs subjected to 90 minutes of ischemia, the maximum concentration was observed after 10 minutes. Recent data have shown that the postconditioning can reduce the subsequent ROS production and lipid peroxidation in isolated myocardial cells. In addition to this, the postconditioning leads to an increase in NO formation, which plays an important role in myocardial protection.

CHAPTER 3

3. CARBONYL STRESS

A common factor of degenerative diseases is oxidative stress, which is a serious imbalance between the production of reactive oxygen species and antioxidant defenses resulting in damage to the main biological macromolecules. In recent years there is a growing interest in carbonyl stress, a condition in which there is an imbalance between the production of glycant applies and the body's defenses against glycation. This condition is considered to negatively contribute to the onset and course of senescence (Xue et al. 2009) and of various diseases, among which diabetes and vascular complications associated with it (Ahmed et al. 2005), renal dysfunction (Agalou et al. 2005) Alzheimer's disease and cardiovascular disease. Protein glycation in recent years is one of the main targets of multiple studies.

3.1 Nomenclature and classification

In 1985, the Nomenclature Committee of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry chose the term glycation to denote all those non-enzymatic and enzymatic reactions that bind a sugar to a protein or a peptide (Sharon 1986). Subsequently, the enzymatic modifications of proteins

and non-enzymatic modification of proteins by saccharides in processes of protein glycation forming glycated proteins (Lis et al. 1993). This eventually led to selective use of the term glycation for non enzymatic modification of proteins by saccharides such that it is now accepted nomenclature. In older scientific literature, one of the most prominent glycated proteins, haemoglobin, has been called glycosylated haemoglobin, non-enzymatic glycosylated haemoglobin, glycohaemoglobin, glucosylated haemoglobin and other variants. Protein glycation involves the non-enzymatic attachment of a reducing sugar or sugar derivative to a protein. The term ‘‘reducing’’ related to the classical activity of saccharides with free aldehyde or ketone groups which reduced Benedict’s solution (Benedict 2002). Glycation is not available to non-reducing oligosaccharides where aldehyde or ketone groups of component monosaccharides have been converted to ketal and acetal groups of glycosidic bonds—such as in sucrose. In its earliest development, protein glycation was thought to be restricted to modification of amino groups of lysine residue side chains and N-terminal amino acid residues. In more recent times, glycation of arginine residues by dicarbonyl metabolites has emerged as a major feature of protein glycation in physiological systems. There is also involvement of cysteine residues. Glycation of proteins occurs by a complex series of sequential and parallel reactions called collectively the Maillard reaction—named after the leading pioneer of glycation research, Louis Camille Maillard (1878–1936). Many different adducts may be formed. In the physiological setting, one of the important saccharides participating in glycation of mammalian

metabolism is glucose, and some of the most important saccaride derivatives are the reactive dicarbonyl metabolites such as methylglyoxal (MG) and glyoxal.

Reactions involved in the process of glycation are classified into early and advanced. In the early phase, glucose reacts with the amino residues of proteins to form initially glycosylamine which then was dehydrated to form a Schiff base. Subsequently, the Schiff base undergoes an Amadori rearrangement to form fructosamine (Hodge 1955). In the next phase, fructosamine is degraded to multiple stable adducts that are called advanced glycation end products (AGEs) (Fig. 3-1).

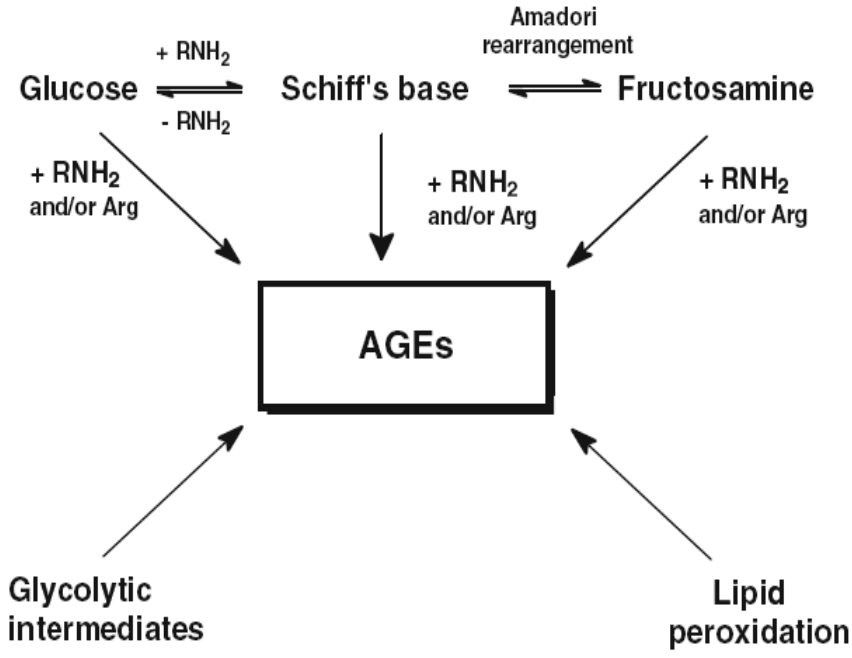

Figure 3-1: *Early protein glycation and AGE formation*

Numerous studies have shown that glucose can be converted into extremely reactive dicarbonyl species, such as α-oxoaldehydes. The α-

oxoaldehydes have the ability to react with proteins forming directly AGEs; it has been observed also that the Schiff bases can be degraded directly in α-oxoaldehyde using a different process excluding the Amadori rearrangement. AGEs are directly formed by spontaneous modification of proteins by reactive dicarbonyl species.These are generated by the degradation of glycolytic intermediates and lipid peroxidation. AGEs are grouped under the name of all the adducts that are produced in the Maillard reaction. Important α-oxoaldehyde or dicarbonyl glycating agents are glyoxal, MG, and 3-deoxyglucosone (3-DG). Therefore, AGEs may be formed in glycation by glucose in pre- and post-Amadori product reactions, and indeed in processes where an Amadori product is not a precursor. AGEs may be formed in both the early and late stages of glycation processes. The term ‘‘advanced glycation endproducts’’ is a misnomer in that AGEs are formed in both early and later, advanced stages of the Maillard reaction.

3.2 Importance of protein glycation

In biology, protein glycation is implicated in the low level endogenous damage to the proteome.

The enzymatic defenses of the organism against glycation consist of enzymes that repair the early glycated proteins and prevent glycation by metabolizing dicarbonyl glycating agents responsible for this process at the physiological level : fructosamine 3- kinase (Delpierre et al. 2000), aldoketo reductase (Baba et al. 2009), and the glyoxalase system (Thornalley 2003). The imbalance between glycating species

and enzymatic defenses against glycation, in favor of the first, is called carbonyl stress. Itis considered in the same way of oxidative stress, and is believed to contribute negatively to aging and diseases (Xue et al. 2009), for example diabetes and its vascular complications (Ahmed et al. 2005), renal dysfunction (Agalou et al. 2005), Alzheimer's and cardiovascular diseases (Ahmed et al. 2005; Rabbani et al. 2005).

3.3 Methylglyoxal: an important glycant agent

At the beginning, methylglyoxal was considered as a toxin or a environmental and/or bacterial metabolite (Cooper 1984). To date, we know that methylglyoxal is formed by spontaneous degradation in physiological systems of triosephosphates, glyceraldehyde 3-phosphate (GA3P) and dihydroxyacetone phosphate (DHAP) in all the organisms that have an anaerobic glycolytic metabolism. The GA3P is approximately 8 times more reactive of DHAP in the degradation to methylglyoxal, but as the ratio between the concentrations of DHAP and GA3P in the cell is about 20, both of these compounds are physiological important sources for the formation of methylglyoxal in situ (Phillips et al. 1993). Other sources of methylglyoxal are considered to be the oxidation of acetone, this reaction is catalyzed by cytochrome P450 2E1 in the catabolism of threonine (Lyles et al. 1992), the degradation of glycated proteins by glucose and the degradation of monosaccharides (Thornalley et al. 1999). In addition to methylglyoxal, another dicarbonyl compound is glyoxal derived

from lipid peroxidation, and from degradation of monosaccharides (Thornalley et al. 1999). The quantification of physiological concentrations of glyoxal and methylglyoxal has been a big issue. Both compounds can be formed through multiple processes such as the degradation of monosaccharides, of glycated proteins and also during the preparation of the samples for the determination of their concentrations. For these reasons, the concentrations are often overestimated. To overcome this reason now methods for α-oxoaldehyde analysis that involve the use of chemical derivatives of such substrates, thus improving the sensitivity of the method are applied. The more recent method involves the use of 1,2-diaminobenzene , to derivatize the substrate, and the detection system with a gas chromatograph associated to mass spectrometer (GC-MS) (Selicharova et al. 2007). Recent studies have shown that the concentrations of glyoxal and methylglyoxal in human plasma are around 100-120 nM, while at the cellular level are 1-5 μM for methylglyoxal and 0.1-1 μM for glioxal (Dobler et al. 2006). In several studies in cell cultures and tissue, concentrations of methylglyoxal used to verify the effect of this metabolite are ten times higher than those considered cytotoxic. Using millimolar methylglyoxal concentrations in the has no relevance to the physiological level (Riboulet-Chavey et al. 2006). The rate of total cellular formation of MG was estimated to be ca. 125 μmol/kg cell mass/day (Thornalley 1988) which for an adult human of 25 kg body cell mass (Ellis et al. 2000) equates to a predicted whole bodyrate of formation of ca. 3 mmol MG/day. As total MG derived glycation

adduct excreted in urine of healthy human subjects was typically <10 μmol/day (Thornalley 2003; Ahmed et al. 2005), it can be inferred that less than 1% MG formed endogenously modifies the proteome. Most of the formed MG (<99%) is metabolised by glyoxalase 1 (Glo1) and aldo–keto reductase (AKRd) isozymes, which thereby constitute an enzymatic defence against MG glycation (see below). MG modifies proteins to form advanced glycation endproduct (AGE) residues. The major AGE formed is the arginine-derived hydroimidazolone Nd-(5-hydro-5-methyl- 4-imidazolon-2-yl)-ornithine (MG-H1) formed via anintermediate dihydroxyimidazolidine. MG-H1 accounts for typically[90% adductstypically 2–4 μM (Phillips et al. 1993; Dobler et al. 2006; Nicolay et al. 2006) and the concentration of MG in human plasma is ca. 100 nM (Beisswenger et al. 1999; Nicolay et al. 2006). Estimates of MG concentrations that are markedly higher than this cannot be sustained given the reactivity of MG with the proteome, the level of MG derived glycation adducts in the steady state and protein turnover (Thornalley 2005; Thornalley 2008). Overestimates are likely due to interferences in analytical methodology particularly degradation of derivatising agent and/or other sample components other than MG during pre-analytic processing. Although the concentration of MG in plasma is ca. 50,000- fold lower than glucose, MG has much higher intrinsic reactivity towards glycation than glucose, 10,000–50,000-fold higher (Thornalley 2005). It is, therefore, predicted that the formation of glycation adduct residues in proteins by MG in vivo occurs at fluxes approaching those of glucose. For the major glycation

adduct of glucose, Fructosyl-Lysine (FL) residues, there is a pathway of de-glycation and thereby repair of FL modified proteins catalysed by fructosamine 3-phosphokinase (Delpierre et al. 2000). Currently, there is no known mechanism of de-glycation of hydroimidazolone-modified proteins. There is, however, slow dynamic reversibility of hydroimidazolone formation with de-glycation half-life of 12 days (Ahmed et al. 2002; Thornalley 2003). This implies that when there is a sustained decrease of the MG concentration, e.g. by induction of increased expression of GLO1, there is expected to be a later commensurate decrease in hydroimidazolone content of proteins. MG-H1 adduct residues are released from proteins by cellular proteolysis. The MG-H1 free adduct thereby formed was the major quantitative glycation free adduct excreted in human and rat urine (Karachalias et al. 2000; Thornalley 2003). Proteins containing MG-H1 residues were predicted to have distorted or damaged structures (Ahmed et al. 2005; Dobler et al. 2006) and therefore may be targeted for proteolysis by the proteasome (Grune et al. 1996; Dudek et al. 2005; Hernebring et al. 2006). Lysosomal proteolysis is also important for degradation of long-lived cellular proteins, endocytosed extracellular proteins (Goldberg et al. 1997) and chaperone- mediated autophagy of cellular proteins (Franch et al. 2001). Release of MG-H1 free adduct from cells and tissues (Thornalley 2003) and decrease of glycated proteins with increased cellular 20S proteasome activity (Hernebring et al. 2006) are consistent with targeting of MG-H1-modified proteins for proteasomal degradation.

3.4 Protein damage by glyoxal and methylglyoxal

Glyoxal and methylglyoxal are considered two powerful reactive species involved in the process of glycation of proteins. The initial state of the process of proteins glycation by glucose leads to the formation of FL and other residues of fructosamine. The final stage of the glycation process provides terminal stable adduct formation (Thornalley 2008). FL slowly degrades to form AGEs; but glyoxal and methylglyoxal may also derive from FL (Thornalley et al. 1999). Owever glyoxal and methylglyoxal directly and quickly react with proteins to form AGEs. For example, by adding 1 μM [C^{14}] methylglyoxal to human plasma "in vitro" and incubating at 37°C an irreversible binding of methylglyoxal to plasma proteins is obtained by 24 h (Thornalley 2005). The most important AGE proteins, from a quantitative point of view, are hydroimidazolones that are obtained by modification of arginine residues by glyoxal and methylglyoxal respectively N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) and N^{δ}-(5-hydro-4-imidazolon-2-yl)-ornithine (G-H1) (Thornalley et al. 2003) (Fig.3-2). Simultaneously also other minor adducts are formed by the modification of lysine residues by the action of glyoxal and methylglyoxal respectively: N^{ε}-(1-carboxyethyl)lysine (CEL) and N^{ε}-(1-carboxymethyl)lysine (CML) and bis(lysil)crosslinks (GOLD e MOLD) (Ahmed et al. 2002). For glyoxal we can include also another adduct derived by modification of

arginine residues (Odani et al. 2001), the corresponding adduct derived from methylglyoxal $N^{(\omega)}$-carboxylmethyl-lisine (CMA) is unstable and not detectable. (fig.3-2)

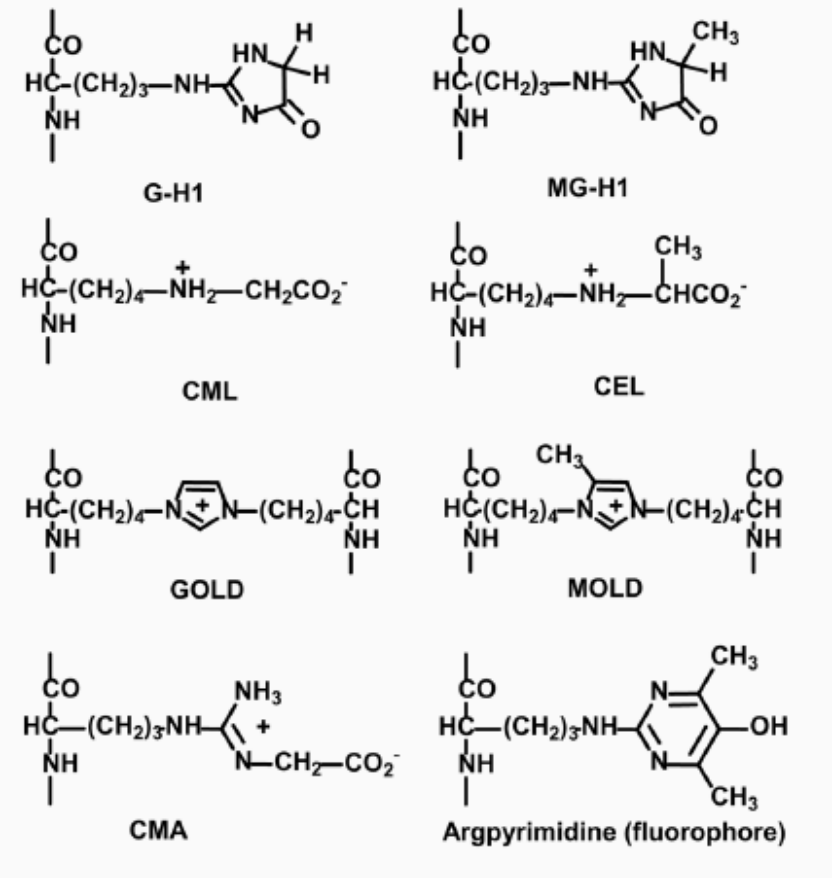

Figure 3-2: *main AGE protein formed by glyoxal and methylglyoxal*

Protein glycation was originally seen as a post-translational modification that primarily affected the extracellular proteins. In particular, it was thought that AGEs were formed slowly during the course of the life and that the AGE concentration was the accumulation of glycation products in the course of existence.

For example, the accumulation of CML and CEL residues was found in the skin (Verzijl et al. 2000). Hydroimidazolones have a lower life span in physiological conditions and their concentration depends on the balance between the amount of adducts that are formed and those that are degraded. The glycated proteins are also formed from short half-life cellular and extracellular proteins . The turnover of these proteins, governed by cellular proteolysis, leads to the release of the so-called glycation free adducts found in urine (Thornalley et al. 2003). The damage caused by protein glycation results in structural changes, these proteins will be degraded by the proteasome (Goldberg 2003). This is an efficient defense process since only a irrelevant portion of these adducts can be found at plasma level. Foods high in sugar and processed at high temperatures are a source of AGEs (Henle 2003). But AGEs present in food proteins have a low bioavailability, in fact less than 10% of these is absorbed (Ahmed et al. 2005). This is due to the resistance of highly glycated food proteins to the proteolysis (Thornalley 2008) and to the fact that some AGEs inhibit intestinal proteases (Oste 1989). The highest concentration of foodborne AGE is in the portal venous blood (Ahmed et al. 2004). AGEs are probably absorbed from foods as free AGEs or glycated peptides. These are easily degradable when absorbed. The most common hydroimidazolones AGE (G-H1, MG-H1, CEL, CML) have a high renal clearance (Thornalley et al. 2003). Therefore, until renal function is optimal, glycation adducts and oxidated proteins absorbed with food, may represent only a minimal risk for the organism, being easily excreted via the urine (Thornalley 2005) (Fig.3-3).

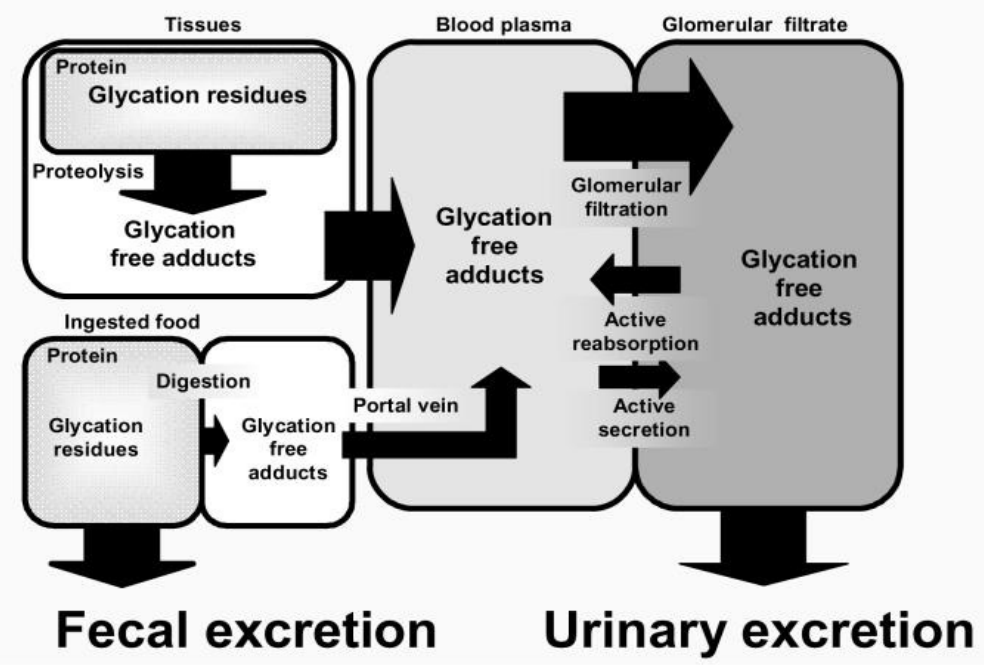

Figure 3-3: *Biodistribution scheme illustrating flows of formation and removal of glycation adducts*

The harmful effects of the glycation process are caused by covalent crosslinks between proteins which confer a resistant effect against proteolysis (DeGroot et al. 2001). The biological impact of protein crosslink can lead to many different effects. Crosslinks of collagen causes a hardening of the vessels and of the joints, while the formation of cataracts is due to a crystalline damage. The proteasome is responsible for the elimination of glycated proteins. The accumulation of damage at the level of the proteasome causes a non-removal and accumulation of damaged proteins, with a consequent negative impact on the body's tissues.

The modification of proteins is detrimental when the amino acid residues are located in sites of protein-protein, enzyme-substrate and

protein-DNA interaction. The most important modification of proteins by methylglyoxal and glyoxal concern the arginine residues.

3.5 The glyoxalase system

The glyoxalase system is present in the cytosol of all the cells and catalyses the conversion of α-oxoaldehydes into the corresponding α-hydroxyacids. Itis composed of two enzymes, glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2) and a catalytic amount of reduced glutathione. In particular, GLO1 catalyses the isomerization of the hemithioacetal, spontaneously derived from the reaction of the α-oxoaldehyde, mehylglyoxal (CH3COCHO) and GSH, to S-D-Lactoylglutathione.

RCOCHO + GSH ⟷ RCOCH(OH)-SG ⟶ RCH(OH)CO-SG

The Km value is 71-130μM and the Kcat is 7-11x 104 min-1.
GLO2 catalyzes the conversion of S-D-Lactoylglutathione in D-Lactate recostituting the GSH consumed in the GLO1-catalyzed reaction.

$RCH(OH)CO\text{-}SG + H_2O \longrightarrow RCH(OH)COO^- + GSH + H^+$

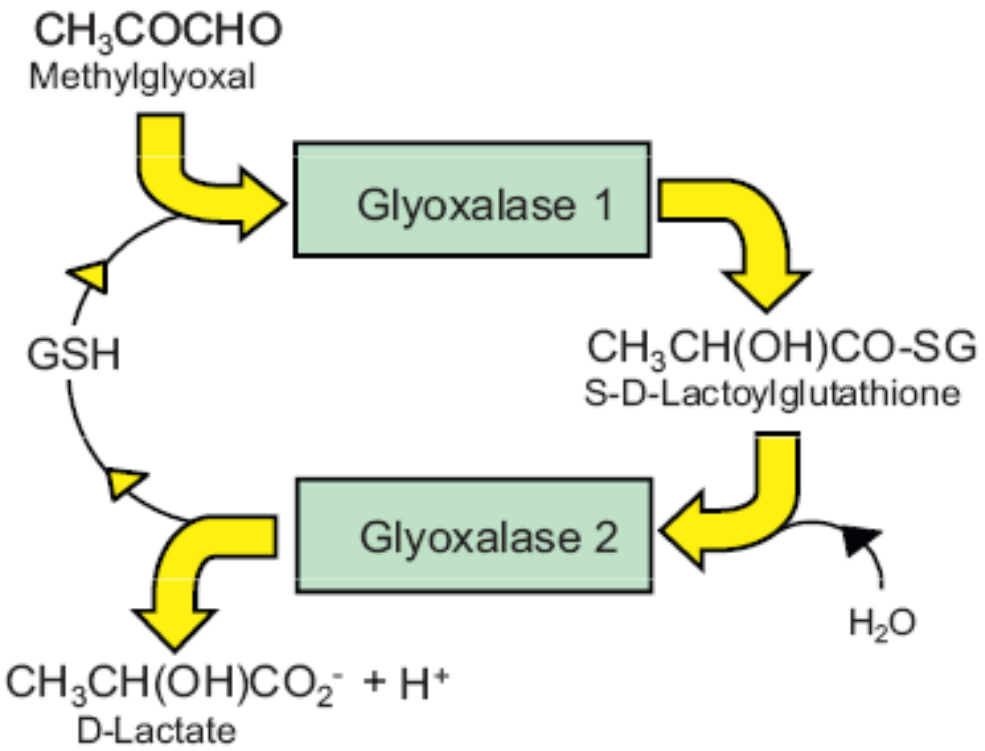

Figure 3-4: *The glyoxalase system*

The major physiological substrates for GLO1 are glyoxal and methylglyoxal; the last one markendly accumulates when GLO1 is inhibited in situ by cell permeable GLO1 inhibitors, by depletion of GSH and by gene silencing of gene encoding for GLO1 (Thornalley et al. 1996; Thornalley et al. 1999). Other substrates are hydroxypyruvaldehyde (HOCH2COCHO) and 4-5-dexovalerate H-COCOCH2CH2CO2H, formed during the reduction of α-ketoglutarate and during the oxidative catabolism of a hee precursor (Grillo et al. 2008). The glyoxalase system, but particularly the action of GLO1 enzyme prevents the increase of these reactive α-oxoaldehydes in cells and thereby suppress the glycation process reactions.

3.5.1 Molecular properties of GLO1

GLO1 enzyme is present in all human tissues, even if his activity in fetal tissues is 3 times higher than this of corresponding adult tissues, where is present at 0,2µg/mg protein concentration. Human GLO1 enzyme is a dimer, expressed at a diallelic genetic locus which encodes for two similar subunits in heteroygotes, and the three isoenzymes are called GLO1-1, GLO1-2 and GLO2-2. All the isoenzymes have molecular mass of 42 kDa (sequence) and 46 kDa (gel filtration) and pI values of 4,8-5,1. The human GLO1 is considered a Zn^{2+} metalloenzyme because each subunits contains one Zn^{2+} ion (Thornalley 1993). GLO1 from Escherichia coli is a Ni^{2+}-metalloenzyme (Clugston et al. 1998).

3.5.2 Structure and functioning of GLO1

184 amino acids are included in the post translation product of the human GLO1 gene. The N-terminal methionine in the post-trascritional mechanism and the N-terminal alanine is stopped by an as-yet-unknown modification. The are 4 sites of phosphorylation. The structure of the complex GLO1 S-benzylglutathione was determined at the 2.2 Å resolution (Cameron et al. 1997) (Fig. 3-5).

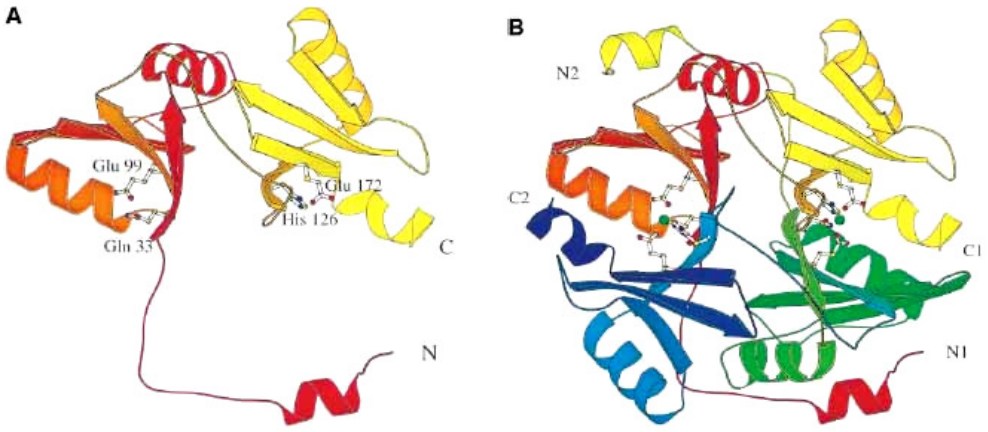

Figure 3-5: representation of the crystal structure of the human in a)monomeric b) dimeric form

Each monomer countains two structurally equivalent domains. The active site is situated at the dimer interface, with the inhibitors and essential Zn^{2+} ion interacting with side chains of both subunits. The zinc binding site involves two structurally equivalent residues from each domain [Gln-33A, Glu-99A, His-126B, Glu-172B] and two water molecules in octahedral co-ordination (Cameron et al. 1997). The reaction catalyzed by GLO1 occurs with a mechanism that involves the proton transfer from C-1 to C-2 of the hemithioacetal, bound to the active site, to form an ene-diol intermediate, followed by a rapid ketonization to the thioester as the final product (Fig.3-6).

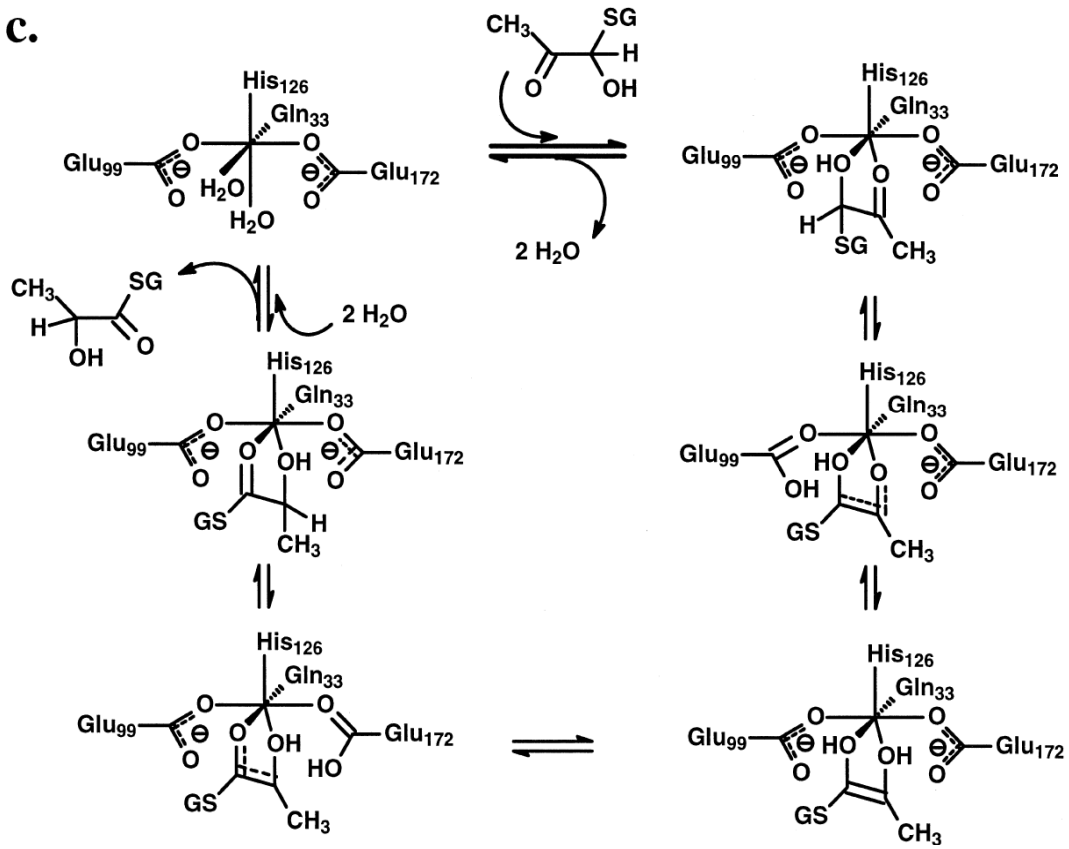

Figure 3-6: Catalytic mechanism of human GLO1 in the isomerization of R- hemithioacetal (Himo et al. 2001)

Both R- and S-forms of the hemithioacetal are bound in the active site of GLO1 and so both are deprotonated; the subsequent reprotonation of the putative ene-diol intermediate occurs stereospecifically to form the R-2-hydroxyacylglutathione derivate. It has been suggested that Glu-99 is the catalytic base for the R-substrate enantiomer and Glu-172 the catalytic base for the S-substrate enantiomer. These two mechanisms lead to the formation of a cis-ene-diol intermediate co-ordinated directly to Zn^{2+} which then reprotonates C-2

stereospecifically to form the R-2-hydroxyacylglutathione (Himo et al. 2001).

methylglyoxal, glyoxal, and hydropyruvaldehyde produces respectively, S-D-lactoylglutathione, S-glycolylglutathione and S-L-glyceroyglutathione, and are hydrolyzed to D-lactate, glycolate and L-glycerate respectively by the GLO2 enzymes (Clelland et al. 1993).

3.5.3 Role of GLO1 in anti-glycation defenses

The formation of methylglyoxal is an intrinsic characteristic of the Embden-Meyerhof pathway or glycolysis; a direct consequence of the presence of triosephosphate, GA3P and DHAP, intermediates in glycolysis. The production of this dycarbonyl metabolite can be minimized by maintaining low triose phosphates concentration. Therefore, the production of methylglyoxal accounts for only 0,1-0,4 % of glucotriose flux.

A peculiarity of methyolglyoxal is the hight reactivity in glycation reactions in vivo, forming AGEs of protein and nucleotides. Methylglyoxal-derivate proteins AGEs are: N^{ε}-carboxyethyl-lysine (CEL), from lysine and the hydroimidazolone derivative of arginine MG-H1 [N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine] and related structural isomers, among with is the predominat argpyrimidine. Nucleotide derived AGEs are: CEdG [N_2-1carboxyethyl)deoxyguanosine], dG-MG {6,7-dihydro-6,7-dihydroxy-6-methyl-imidazo-[2,3-b]purine-9(8)one}, dG-MG_2 [N_2-(1-hydroxy-2-oxopropyl)deoxyguanosine] (Thornalley 2003). Probably glyoxal and other GLO1 substrates may give rise to similar adducts. The

glyoxalase system is an efficient enzymatic detoxification system suppressing the formation of AGEs originated by methylglyoxal and glyoxal. Recently, glycation adducts of cellular and extracellular proteins were quantified. These studies have shown that 0,1-2% of total cellular arginine is modified by methylglyoxal, the highest estimate being in human lens proteins of elderly subjects with low GLO1 activity (Ahmed et al. 2003).

The experimental evidence that have allowed to demonstrate the role of GLO1 in preventing the formation of AGEs derived from studies of normoglycaemic and hyperglycaemic endothelial cells culture. Hyperglycaemia increases concentration of methylglyoxal, D-lactate and cellular protein AGEs. GLO1 overexpression counteracted the increase in methylglyoxal and cellular protein AGEs, and increased D-lactate concentration. This indicated that GLO1 has an important role in suppressing the formation of protein AGEs (Shinohara et al. 1998), preventing cell dysfunction and cell aging. GLO1 is a GSH-dependent enzyme. Under physiological conditions, the rate of hemithioacetal hydrolysis to GSH and methylglyoxal is of 10^3 times faster than the rate of isomerization by GLO1. This implies that "in vivo" there is a rapid pre-equilibrium between GSH, methylglyoxal and the hemithioacetal, and the activity of GLO1 is proportional to the amount of available GSH level. Cytotoxic effects caused by the accumulation of methylglyoxal and to a lesser extent of glyoxal, are due to the depletion of GSH by oxidative and non-oxidative mechanisms. This indicates that methylglyoxal predominantly accumulates in conditions of oxidative stress and has the ability to contribute to the cytotoxicity

induced by oxidants (Abordo et al. 1999). The other accumulation of the highly reactive metabolites must be limited. For this reason it is capital the role of GLO1 that serves as a detoxification system toward these dycarbonilyc compounds and therefore acts as an integral part of the defenses of the organism against enzymatic glycation. In diseases states, such as microbial infections and cancer the target is to induce apoptosis of microbial organisms and tumour cells . In these conditions, the antitumor and antimicrobial activity were obtained through the use of cell-permeable GLO1 inhibitors. S-p-bromobenzylglutathione (SpBrBzGSH) is a potent inhibitor of human GLO1. Diesterification of this inhibitor protects this GSH conjugate against extracellular degradation by γ-glumyl transpeptidase and makes it cell permeable, therefore able to cross biological membranes. In the cell cytosol, SpBrBzGSH diesters are de-esterified and GLO1 is inhibited. S-p-bromobenzylglutathione-cyclopentyl diester showed a potent antitumor effect both “in vitro” and “in vivo” (Thornalley et al. 1996).

3.5.4 Role of methylglyoxal in the GLO1 regulation

GLO1 substrates, methylglyoxal and glyoxal, leading to modifications of proteins, nucleotides and other biological macromolecules are very harmful to the organism. Our organism to counteract these damaging effects presents the glyoxalase system, composed by GLO1 and GLO2, that catalyzes the conversion of α-oxoaldehydes to the respective α-hydroxyacids (Thornalley 2003). Recent studies show

that GLO1 activity is regulated by GLO1 substrates and the process of glycation may have a functional role in the signal transduction pathway. A high GLO1 activity is required, in conjunction with a high flow of methylglyoxal associated with an intense flow of triose phosphates and anaerobic glycolytic activity, to protect the proteome and the genome respectively from functional and mutational damage.

Secondly, the activation of the AGEs receptors (RAGE) by S100 proteins found in increasing concentration in human plasma in inflammation, is associated with the decrease of the GLO1 expression. The induction of diabetes in wild type mice decreased the expression of GLO1, while the induction of diabetes in RAGE-deficient mice had no effect on the expression of the enzyme (Bierhaus et al. 2006). The reduction of the GLO1 expression leads consequently to an increase of glycation (Thornalley 2003). AGEs were co-localized mainly at the level of RAGEs. This may be due to the activation of RAGEs by S100 proteins, by the local reduction of the GLO1 expression and therefore by the increase in AGE formation at the tissue level. This can be part of an inflammatory response that leads tomodify proteins with dicarbonyl-hydroimidazoline residues and route them to the proteasome for destruction.

The proteins modified by MG then become the target for the destruction by the proteasome (Du et al. 2006). Finally, GLO1 can be phosphorylated and the glycation of proteins may increase in case of apoptosis induced by tumor necrosis factor (αTNF). The regulation of GLO1 by methylglyoxal suggest a possible signaling role carried out by the glycation of proteins and nucleotides.

This role includes the development of malignant transformation (Thornalley 2003), cell death through the activation of apoptotic processes, erythrocyte apoptosis (Dobler et al. 2006) and activation of acute and/or chronic inflammatory processes.

3.6 Effect of glycation in pathological conditions

The accumulation of the adducts of glycation is associated with enzyme inactivation, protein denaturation and cell-mediated immune response. The excessive glycation of nucleotides is related to mutagenicity and apoptosis, whereas the excessive glycation of membrane phospholipids in lipid peroxidation and destruction of the phospholipid bilayer (Thornalley 2008). The body has a detoxification system that protects against cell and tissue damage induced by glycation (Thornalley 2003). This system is imperfect ; under normal physiological conditions, the adducts of glycation of proteins, nucleotides and phospholipids are generated spontaneously, but in small quantities.

Imately approx 0.1-1 % of the lysine and arginine residues of proteins, one of 10^5 nucleotides at the DNA level and 0.1% of the basic phospholipids of membranes are modified. Glycation adducts of proteins are removed by proteolytic enzymes at the level of proteasomes and lysosomes (Westwood et al. 1997). Glycation adducts of nucleotides are removed by repair systems DNA to excise nucleotides (Murata-Kamiya et al. 1999), and finally the AGEs of the phospholipids are removed by the lipid turnover (Requena et al. 1997). However, the metabolism of these glycation adducts leads to

the genesis of additional products that are called glycation free adducts that are excreted primarily in the urine, along with glycation adducts taken with food. The enzyme system counteracting the glycation is overwhelmed in some disease states, such as diabetes and renal dysfunction, resulting in accumulation of glycation products. A strong decline of the activity of GLO1 in aging was shown, so as to assume its involvement in physio-pathogenesis of senescence (Morcos et al. 2008). The main mechanism to remove glycation products from blood is the urinary excretion of the resulting metabolites, hence the marked accumulation of glycation free adducts in the case of clinical or experimental uremia (Rabbani et al. 2007). Even the oxidative stress is related to the glycation because of the depletion of GSH and NADPH that occur in oxidative stress, leading to a reduction in situ of the GLO1 activity (Abordo et al. 1999) and, consequently, an increase in the concentrations of glyoxal and methylglyoxal and glycation reactions.

At the same time, also the glycated proteins by α-oxoaldehides may contribute to oxidative stress, increasing the proportion of ROS such as superoxide anion, hydrogen peroxide and the hydroxyl radical. Glycation contributes to morbidity and mortality associated with high social impact diseases such as diabetes, cardiovascular diseases and kidney dysfunction, and seems to contribute in a determinant manner to the onset and course of others, such as Alzheimer's diseases, rheumatoid arthritis and senescence (Thornalley 2008).

3.6.1 Glycation in cancer

Several studies have shown that the use of GLO1 inhibitors as SpBrBzGSH leads to an accumulation of methylglyoxal and glyoxal, an increase in the nucleotides glycation and induction of apoptosis in cancer cells (Thornalley et al. 1996). It is not yet clear which is the mechanism by which these GLO1 substrates induce apoptosis, although it has been demonstrated the involvement of JNK and p38 protein kinases (Sakamoto et al. 2001).

It has also been shown that fibroblasts, overexpressing GLO1, are resistant to treatment with anticancer drugs such as doxorubicin and mitomycin C (Ranganathan et al. 1995). The GLO1 overexpression can, therefore, confer drug resistance against anticancer therapies. Accordingly, the use of inhibitors of GLO1 could have a twofold effect: to promote the glycation level of tumor cells by inducing apoptosis and reduce drug resistance by increasing the effectiveness of anticancer drugs.

3.6.2 Role of AGE in ageing

The increase in protein damage in aging was determined by analysis of damage markers to long biological half-life proteins : typically it is absorbed the proteins the crystalline lens, which undergo a limited degradation by the proteasome (Bloemendal et al. 2004), and skin and joints collagen. These have respectively a 14.8 and 115 years half-life (Verzijl et al. 2000). The AGEs content in the crystalline lens increases significantly with the age increase of the subject (Ahmed et

al. 2003). Furthermore, an increase in the amount of CML and CEL was detected in fibroblasts of elderly subjects (Gonzalez-Dosal et al. 2006). Finally, the content of CML and CEL in skin and joints collagen increases with advancing age (Verzijl et al. 2000).

Studies in rats showed that the content of CML in skin collagen increases significantly with age (Cefalu et al. 1995).

The immunoblotting analysis of skeletal muscle tissue of rats with anti -AGE 6D12 able to recognize residues of CML and CEL shows an increase of the immunoreactivity of the β-enolase enzyme with aging (Snow et al. 2007);. Ageing in Wistar rats increases the content of the CML residues of, but in the same animals, a similar increase of CEL residues in cardiac mitochondrial proteins was not found (Pamplona et al. 2002). The amount of CEL and CML in proteins of *Drosophila melanogaster* increases with age and is significantly higher in insects kept at 27 °C compared to those maintained at 18 °C, in which the mortality was significantly lower (Jacobson et al. 2010). In the *C. elegans* nematode, an accumulation with advancing age of the MH- H1 residues in mitochondrial proteins was abserved, and this process was, however, opposed and prevented by the overexpression of the GLO1 enzyme. GLO1 overexpression was able to decrease the glycation proteins induced by reactive species as methylglyoxal and glyoxal, reducing the amount of MG -H1, G-H1 and CEL and helping to maintain lower levels of markers of oxidative damage. The prevention of oxidative stress induced by GLO1 overexpression is associated with a reduction of the structural changes of mitochondrial proteins by AGEs and thus prevent potential mitochondrial

dysfunction and concomitant increase in the formation of ROS in aged nematodes (Morcos et al. 2008).

3.6.3 Role of AGE in cardiovascular diseases

Increased concentrations of methylglyoxal in hypertension were first suggested by studies of aortic smooth muscle cells (VSMCs) obtained from Wistar-Kyoto rats clinically hypertensive and from normotensive control rats. The concentrations of methylglyoxal measured in the aortic VSMCs and in the kidney (Wang et al. 2004), have appeared in all cases overestimated of about 10-100 times compared to the expected data. This can be attributed to the intrinsic formation of methylglyoxal during the processing of the samples; assaying the amount of this compound with the most reliable methods were found to be significantly lower MG values, 2 μM in rat blood and 5 pmol / mg of protein in rat liver (Phillips et al. 1993). The "in vivo" accumulation of AGEs over time contributes to changes in the structure and function of the cardiovascular system and causesarterial stiffening, myocardial relaxation abnormalities, atherosclerotic plaque formation and endothelial dysfunction. One of the proposed mechanisms includes additional cross-linking of collagen by glycation of its free amino acids. The collagen-AGEs cross-linking will produce stiffness of blood vessels. Sims et al. in a histological study on 27 samples of post-mortem aortas from people with diabetes, found a relationship between AGEs accumulation and aortic stiffness (Ahmed et al. 2005). AGEs have also the ability to damage the cardiovascular

system through the reduction of LDL uptake by cell receptors. This mechanism involves the glycation of the LDL particle on the apolipoprotein B and in the phospholipid components of LDL. The glycated LDL is more susceptible to cross-linking with collagen on the arterial wall than non-glycated LDL, and it is not taken up into the cell and accumulates. Macrophages uptake of these modified LDL lead to foam cell formation, and the development of atheroma (Agalou et al. 2005; Hambsch et al. 2010). Another important study showed the role of increased androgens during and after menopause as a risk factor for cardiovascular damage in women, with an associated increase in AGEs. A study in 106 postmenopausal women found significant correlations between testosterone and free androgen levels and AGEs after adjustment for age, body mass index, insulin resistance indices, and fasting glucose and insulin levels (Phillips et al. 1993).

In a patient with renal dysfunction and a rare GLO1 deficiency frequent CVD events occured, although no risk factors for these diseases were present (Miyata et al. 2001).

In patients undergoing hemodialysis, the A419C polymorphism for the GLO1 gene was associated with an increased risk of CVD complications (Kalousova et al. 2008). In addition, some studies have shown a high rate of mortality in patients homozygous for the 419CC GLO1 gene mutation (Kalousova et al. 2010). The analysis of GLO1 single nucleotide polymorphisms (SNPs) showed that the most common SNPs are in -7 (C or T) and 20203 (C or A) positions with respect to the start site of translation.

3.6.5 Role of AGEs in atherosclerosis and hypertension

AGEs that are formed at low concentration contribute to the regulation of the physiological process of tissue remodeling , but can be detrimental when their production becomes abnormal with respect to anti-glycant defenses of the organism (Stitt et al. 2004). It was also suggested that some AGEs play a role in normal biological functions, while others, defined precisely AGE toxic, play a pathological role (Takeuchi et al. 2004). While these specific points are still unclear, there are more and more confirmations showing that AGEs are implicated in diseases such as atherosclerosis and hypertension (Wu 2006).

Studies on diabetes, a condition associated with insulin resistance with a high incidence of vascular complications, provide substantial evidence of the implication of AGEs in atherosclerosis. The concentrations of AGEs were elevated in both diabetic patients and in animal models of diabetes, and are associated with the changes observed in atherosclerosis (Fosmark et al. 2006). The research also shows that treatments reducing AGEs or blocking the RAGE attenuate these vascular changes (Alderson et al. 2003). Individuals not suffering from diabetes, but with atherosclerosis, have high levels of the protein AGE-apo B in the serum (Stitt et al. 1997), and AGEs have been identified in atherosclerotic lesions in animal models of rabbit (Palinski et al. 1995) or in humans (Sima et al. 2002). Studies conducted “in vivo” on hypertensive rats and on rats having diabetes-induced hypertension show that MG and AGEs levels are elevated

(Wang et al. 2005). MG administered in the diet to Wistar-Kyoto rats (WKY) causes an increase in AGE at the tissue level and is responsible for the onset of hypertension (Vasdev et al. 1998). In a study conducted in female subjects with pre-eclampsia, a condition of high blood pressure in pregnancy, the expression of RAGE is increased in vascular tissues (Cooke et al. 2003). In patients with essential hypertension, the levels of soluble RAGE, which neutralize AGEs, are inversely correlated with blood pressure levels (Geroldi et al. 2005). AGEs tend to form crosslinks in the proteins and in particular bind to the long-lived proteins such as collagen or elastin (Aronson 2003). This contributes to vascular stiffening. Recently it has been shown that the increase of AGE concentration in plasma is associated with an increase of vascular stiffness in patients suffering of not treated essential hypertension (Brownlee 2001). Such an increase in vascular stiffness and the resulting systolic hypertension dramatically increase cardiovascular risk (Safar 2001). This situation is very common in aging, but can be accelerated in diabetes or in other conditions where the formation of AGEs is increased (Schram et al. 2005). Agents that contribute to the breaking of crosslinks formed by AGE proteins showed the ability to reduce the cross-linking and hardening of the arteries in animal models of diabetes (Wolffenbuttel et al. 1998), to improve vascular compliance in elderly subjects with atherosclerosis (Kass et al. 2001), to improve endothelial function (Zieman et al. 2007) and lower blood pressure in patients with systolic hypertension (Bakris et al. 2004). The role of AGEs in hypertension was also corroborated by animal studies showing that treatments that

target AGEs are able to reduce blood pressure (Midaoui et al. 2003). Other research on AGEs show that such glycation adducts not only alter the structure but also the functionality of proteinaa which bind irreversibly, leading to changes typical of hypertension and atherosclerosis, including increased oxidative stress (Zhang et al. 2006), endothelial cells disfunctions (Wautier et al. 2004), alteration of the reserves of calcium (Jan et al. 2005), onset of inflammatory processes (Kislinger et al. 2001) and changes in signal transduction pathways (Cohen et al. 2003).

3.6.5 AGE and neurodegenerative diseases

The processes underlying neurodegeneration are complex and may involve several aspects, such as excitotoxicity, mitochondrial dysfunction, abnormal protein aggregation and inflammation (Nicole et al. 1998). It has been hypothesized that the formation of ROS is a primary consequence only in certain aspects of the progression of neurodegeneration. In fact, numerous studies have shown that, in addition to ROS, reactive carbonyls as MG could contribute to neurodegeneration (Picklo et al. 2002), subjecting the cells to carbonyl stress characterized by an imbalance between accumulation and detoxification AGEs

Glycation and AGEs, as noted in previous chapters, contribute to post-translational modification of proteins. AGEs have been linked to the development of degenerative diseases including cataracts, and diabetic complications and AD. In AD, AGEs are accumulated in a age dependent manner and according to the stage of disease in neurons

and microglia, and β -amyloid plaques, indicating that under these conditions there is an imbalance between the formation and degradation of proteins modified by AGEs (Luth et al. 2005).

Among the various reactive carbonyl compounds and precursors of AGEs, MG is probably the major contributor to the intracellular formation of AGEs, as it is extremely consistently produced by the degradation of triosephosphates. MG concentration increases in specific pathophysiological conditions, for example, when the levels of triosi phosphates are high, when GSH concentration, cofactordetermining the speed of gliossalasi I, or is low, when the levels of GLO1 expression are reduced, as it has been described in neurons and microglia in people older than 55 years (Kuhla et al. 2007).

Although the exact intracellular MG concentration in neurons is not yet known, concentrations up to 300 mM were measured in CHO (Chinese hamster ovary) cell culture (Chaplen et al. 1998).

The AD brain is characterized by two types of protein aggregates : extracellular plaques, which consist mainly of β-amyloid (Aß), and intracellular neurofibrillary tangles (NFTs), predominantly composed of tau protein, protein associated with the microtubule (MAP-tau). Both proteins, Aβ and MAP-tau, are modified by AGEs (Yan et al. 1994). Other proteins, not yet identified, are modified by AGEs, and gradually accumulated in the cortical areas of the brain in the advanced stages of AD (Luth et al. 2005). As a result of the formation of AGEs, the proteins are heterogeneously modified in their side

chains, polymerize, thus becoming more insoluble and resistant to the action of proteases.

Although several studies have shown that MG induces apoptosis in neuronal cells and not, probably through the activation of the family of the mitogen-activated protein kinase kinase or c-jun NH2-terminal (Kuhla et al. 2006), the molecular mechanisms underlying MG cytotoxicity remain poorly understood. In a study of proteomics in the human neuroblastoma cell line SH-SY5Y , it has been shown that MG modifies the expression of several proteins that probably play a role in neurodegeneration . Another study examined samples of cerebrospinal fluid (CSF) from six AD patients, assessed by the Mini-Mental State Examination (MMSE), and six CSF samples from healthy controls. Weak increases in the mean values of free MG (22.1 ± 20.2 nM) in the group of patients compared to healthy controls (10.1 ± 5.2 nM) were observed but without statistical significance. Differences in the concentration of glyoxal between the two groups were not found (Kuhla et al. 2005). However, the concentrations of both compounds were about 5 to 7 times higher than that of human plasma samples (Odani et al. 1999), probably because the protein content in the CSF is much lower than in plasma, and then CSF proteins does not react completely with MG.

Although the degree of protein modification by AGEs in senile plaques has not been quantified, it is probably much higher than that of plasma proteins, given that the half-life of proteins in senile plaques is estimated to be about 30 years compared to the much shorter-half of1-2 months of most of the plasma proteins.

It has been proposed that AGEs transmit their signals through the RAGE (Schmidt et al. 1994), which is a member of the cell surface receptors of the immunoglobulin superfamily (Schmidt et al. 2000). Its series of ligands include AGEs, anfoterine, the S100 calgranuline and fibrillar peptides including Aβ in the brain suffering from AD (Yan et al. 1998). Both AGE and Aß are present in senile plaques (Wong et al. 2001) and has been shown to up-regulate proinflammatory cytokines through RAGE and via NFkB-dependent pathway (Schmidt et al. 2000). This led to the conclusion that the Aβ-RAGE interaction plays an important role in the pathophysiology of AD.

It has been shown that both AGEs and Aß lead to a reduction of ATP levels in a dose dependent manner. Because ATP is important for many neuronal functions, including the maintenance of gradients of Na + / K +, low levels of ATP in the neurons of AD patients (caused by AGE and Aß) may directly influence the synaptic transmission and create the clinical picture of dementia (Kuhla et al. 2004).

CHAPTER 4

4. Glucosinolates and Sulforaphane

4.1 Chemical structure and properties

Glucosinolates (GLS), an important phytocomponent group, are present at high levels in plants of the Cruciferae family, particularly Brassica, such as broccoli, cabbage and Brussels sprouts. Although abound 120 molecules have been identified, each plant contains up to 4 different GLS in significant quantities (Fahey et al. 2001). In plants, the GLS and their metabolites have fungicidal and bactericidal properties. Their composition in plants varies due to many factors: the climate, the type of crop, the various treatments to which they are subjected in different stages of collection, storage, the preparation and consumption. From the biological point of view, they are a class of inactive compounds until they have been enzymatically hydrolysed to various bioactive break down products by the endogenous plant enzyme myrosinase. GLSs are (*Z*)-*N*-hydroximinosulfate esters, possessing a sulfur-linked β-D-glucopyranose moiety and an amino acid-derived side chain (Fig. 4-1). Side chain and sulfate group have an anti stereochemical configuration across the C =N double bond. The structure of the side chain is highly variable and may possess aliphatic (alkyl, alkenyl, hydroxyalkenyl, ω-methylthioalkyl, ω-

sulfinyl-, and ω-sulfonylalkyl), aromatic (benzyl, substituted benzyl) or heterocyclic (indolyl) groups. The majority of the currently known GLSs may be subdivided according to their hydrolysis products into aliphatic and aromatic, terminally-unsaturated, β-hydroxy-, and indolyl GLSs, in which the first group includes a wide range of homologues (n = 3 to n = 11). A chemical classification of all so far discovered GLSs is reviewed by (Fahey et al. 2001). The presence of the sulfate group in the molecule confers strongly acidic properties on intact GLSs (Fig.4-2). Thus, they are non-volatile and occur as salts with the GLS anion usually counterbalanced by potassium.

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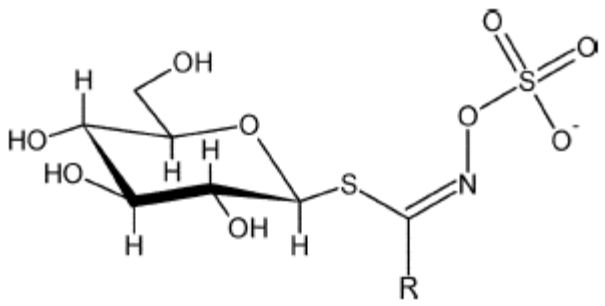


Figure 4-1: *General structure of the GLS*

Many of the biological effects at the base of the health properties of these vegetables are attributed not to the GLS but to their hydrolysis products. The hydrolysis reaction is catalyzed by myrosinase enzyme which is located in separate compartments within the plant tissues, and takes place when, due to the disintegration of the tissues, the enzyme is in contact with the substrate (Cottaz et al. 1996). The GLS are present in all parts of the plant, but with quantitative and qualitative differences, for example in the flowers the total amount

may be 10 times higher than in other plant tissues and represent 10% of the total (Holst et al. 2004)

Myrosinase is a thioglucoside glucohydrolase that hydrolyzes the glucosinolate molecule, leading to the formation of glucose, sulfate and unstable aglycone, thyohydroximate-O-sulfonate. The last one undergoes a spontaneous rearrangement which leads to a wide variety of products, whose chemical structure depends on the initial side chain of the GLS and the reaction conditions (Ludikhuyze et al. 2000) (Fig.4-2). At pH 6-7 the most common products are isothiocyanates (ITC). Among them there is a compound that has attracted the attention of researchers. This compound is sulforaphane (SF) [4 - (methylsulfinyl) butyl - isothiocyanate], produced by the hydrolysis of the corresponding glucosinolate, the glucoraphanin, which has been studied for its anti-inflammatory and chemopreventive properties.

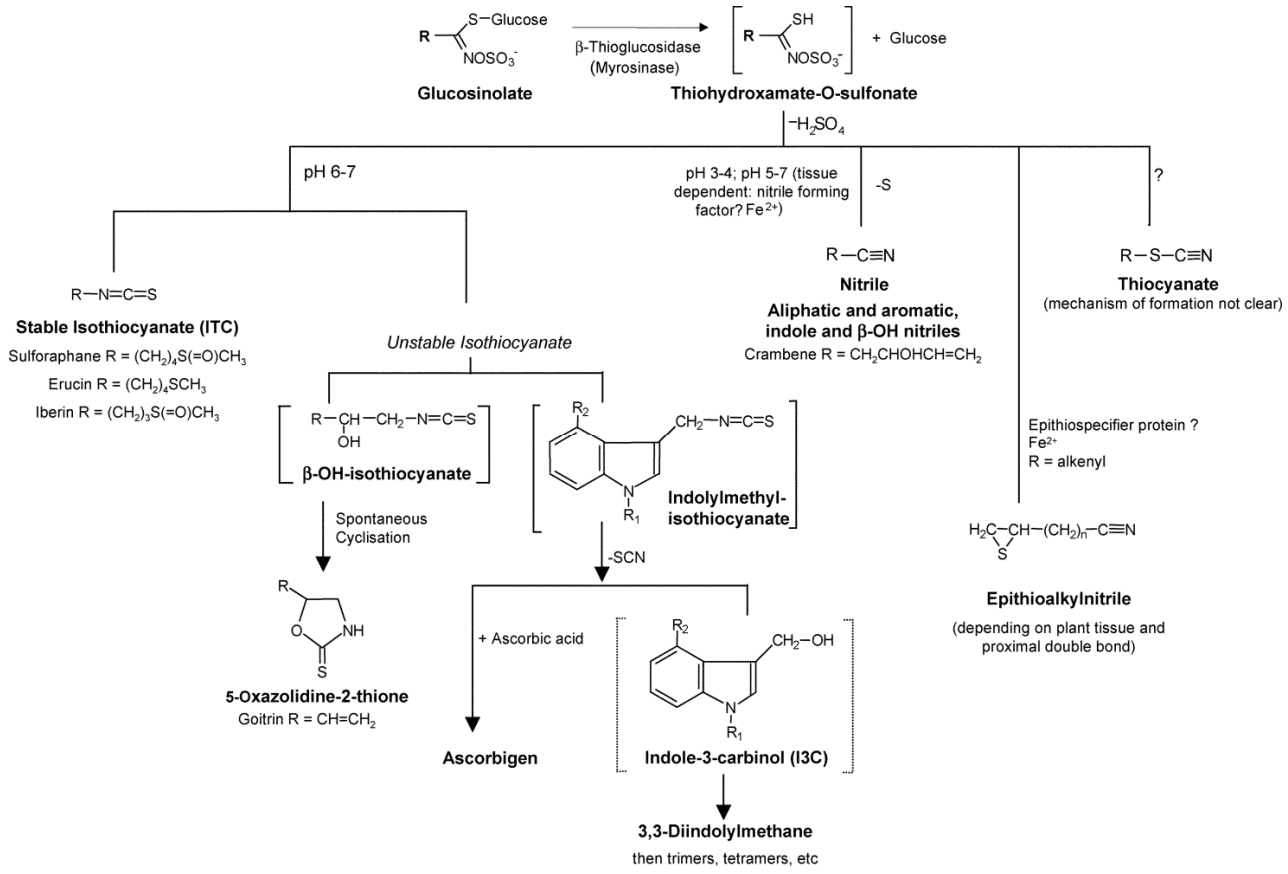


Figure 4-2: Products of GLS hydrolysis

4.2 Metabolism of glucosinolates

Understanding of factors, that limit the release of phytochemicals from the food matrix, the degree of absorption and their fate in the body, is essential to determine their mechanisms of action and their role in maintaining health. The term "bioavailability" describes all these aspects. It was coined by the Food and Drug Administration (FDA) as the amount to which a functional group with therapeutic action is absorbed and becomes available at the site of action. The concept of bioavailability includes:

1. The release and dissolution of a compound to be subsequently absorbed (bioaccessibility)
2. The absorption
3. The tissue distribution
4. The metabolism
5. The excretion

These phases are represented in figure 4-3.

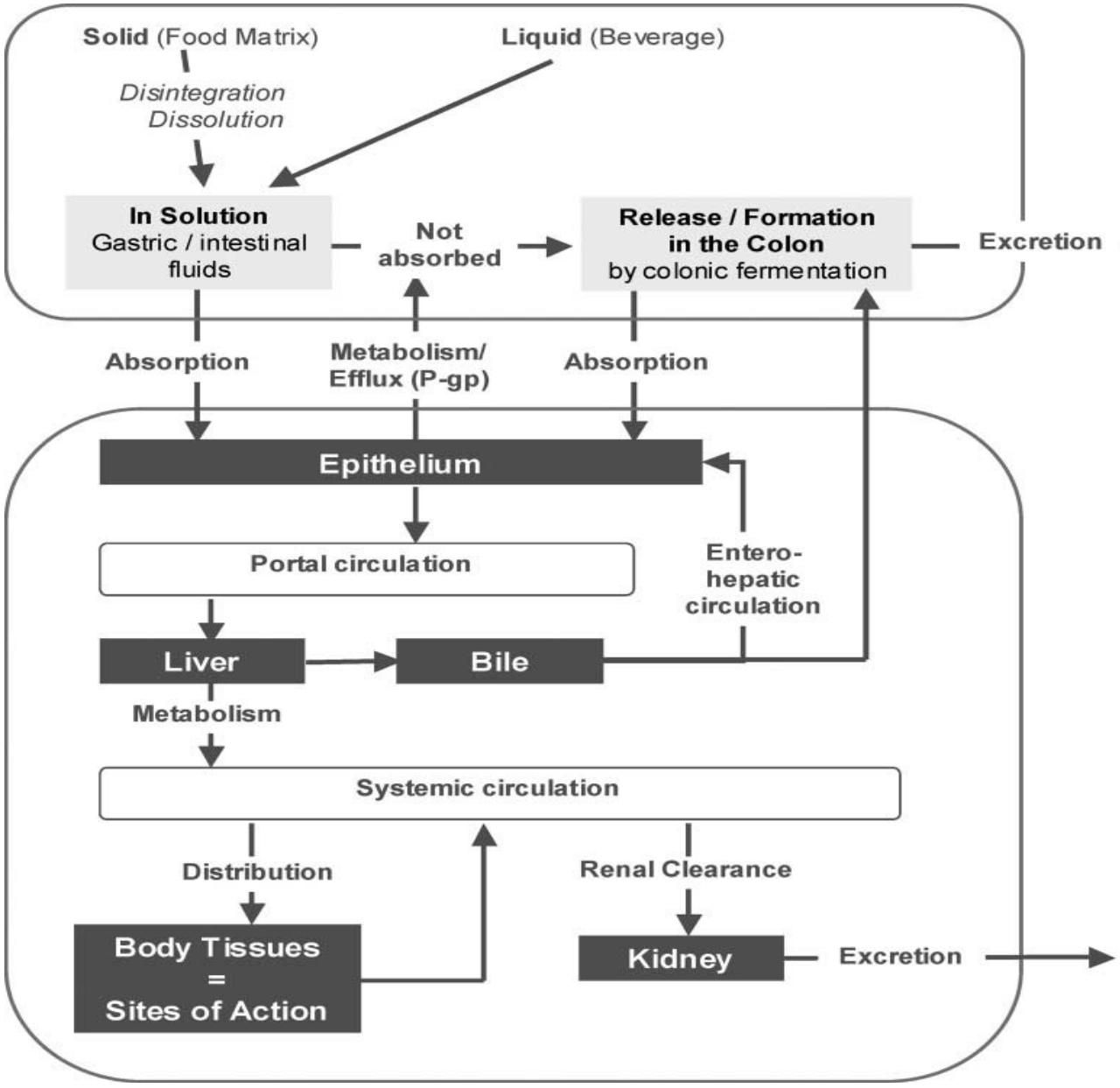


Figure 4-3: Path of phytocomponents through the organism

4.2.1 Release and absorption

The term release means the release of the compound from the food matrix and its dissolution in biological fluids. These steps are crucial for the mixture arrives to the surface of the intestinal epithelium in the most appropriate form to be absorbed by enterocytes or to cross the epithelial layer through the cell junctions.

The release from the food matrix and the formation of hydrolysis products of GLS depends on several factors :

1. GLS Concentration in plants
2. Hydrolysis due to the damage of plant tissues
3. Physical and chemical characteristics of the GLS and the corresponding ITC and their stability
4. Level of rupture of the tissues during mastication
5. Digestion

In addition to the concentration of GLS and food treatments also the matrix affects the release and absorption (Conaway et al. 2000).

The dissolution of a compound is determined by its solubility in an aqueous environment, by the ionization (pKa) and the lipophilicity that is defined by log P. This is the octanol-water partition coefficient, which is a crucial factor for the partition to the side of the plasma membrane. To obtain optimal absorption at the intestinal level the value of log P should be in a range from 0.5 to 2.0. The GLS would be able to cross the cell membrane through a carrier or through aqueous pores. The value log P value, for the GLS degradation products, not depends on the structure of the side chain and varies from 0:23 to 4:37 (Cooper et al. 1997). The presence of a glucoside group in the molecule can indicate an active transport through the glucose transporter, but in vitro studies have shown that this does not happen. A facilitated transport could be used. The GLS degradation products, compared to the parent compounds, are much more lipophilic and have a lower molecular weight, so their absorption can take place, at least in part, by passive diffusion. SF has a log P value of 0.72 and a

molecular weight of 177 g / mol (Cooper et al. 1997).The ITC are conjugated with GSH by the glutathione-S-transferase enzyme. It was observed that hight amounts of GSH within the cell leads to an increase in ITC cell uptake.

4.2.2 Distibution

The term distribution means the path of a compound from the site of administration to the vessels and tissues. The parameters that influence the distribution of a compound in the body are: blood flow, the presence of membranes, the affinity for tissues and binding to plasma proteins, such as albumin and glycoproteins. Only the unbound fraction to these proteins can pass through the membranes. Recently, some researchers have developed a sensitive and appropriate method which allows the determination of low concentrations of ITC and metabolites in biological fluids and tissues (Ye et al. 2002). They observed that following a rapid absorption and appearance in plasma, the ITC peak levels (0.94 ± 2.27 mol L-1) from broccoli sprouts (especially sulforaphane), decrease. These data demonstrate a rapid distribution, which follows a first order kinetics with a half-life of 1.77 ± 0:13 hours (Ye et al. 2002). ITC are transported in the blood bound to plasma proteins to which they bind strongly presumably by interaction with -SH groups, and only in small part, in free form. The distribution to tissues involves the passage of the compounds through the membranes and this follows the same uptake mechanism described for intestinal absorption. The driving

force for ITC passive diffusion across membranes is its conjugation with GSH. A study, carried out by administering to rats ^{14}C ITC, revealed high levels of ITC in the stomach and intestine, intermediate concentrations are found in the pancreas and spleen and very low at the level of heart and brain (Conaway et al. 1999) .

4.2.3 Metabolism

The bioavailability of a compound, even if absorbed in high concentrations, may be limited by a rapid and extensive metabolism. It is therefore important to determine which are the metabolites of this compound and in what concentrations they are found in blood, urine or faeces.The GLS are not present in the faeces, therefore these compounds are subjected to an extensive metabolism. As already said, the GLS are hydrolyzed to ITCs by the myrosinase enzyme in the oral cavity as a result of liberation of the enzyme from the vegetable matrix, or at intestinal level by the microbiota. The first step in the metabolism of ITCs is the conjugation with GSH. It is not clear if this conjugation occurs in the duodenum during absorption, or in the liver, which is the site with the highest GST. Subsequently, the ITC conjugates are metabolized by different enzymes such as the glutamyltranspeptidase (GTP), the cysteinylglycinase (CGase) and N-acetyltransferase (NAT) that lead to the formation of the different metabolites shown in Figure 4-4.This sequence of reactions usually leads to the formation of mercapturic acid, a hydrophilic metabolite that can easily be excreted in the urine.

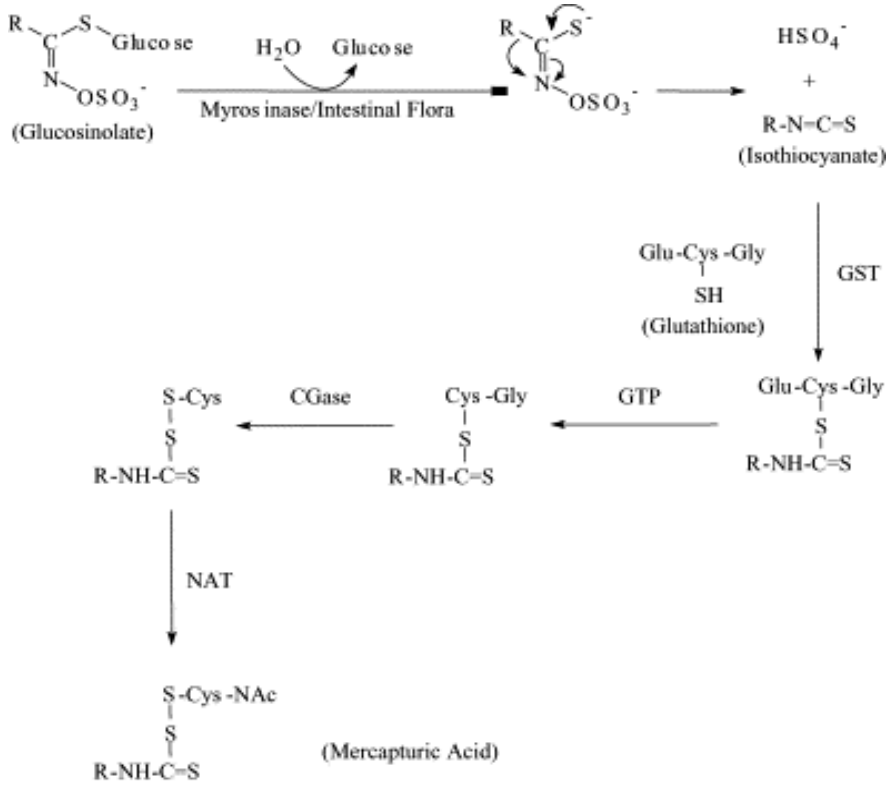


Figure 4-4: *GLS metabolites*

The main metabolites of urinary excretion in humans are N-acetyl cysteine conjugates (NAC) that are currently used as reliable biomarkers of exposure to GLS (Shapiro et al. 2001).

4.3 Absorption of dietary ITC

In agreement with the observation that ITC are readily absorbed and metabolized in vivo mainly through the way of the mercapturic acid and then excreted in the urine as NAC, numerous studies have shown that dietary intake of ITC can be assessed by measurement of the NAC conjugates in urine, which are then to be a reliable marker of exposure.

Ye et al.(2002) demonstrated in a study conducted on volunteers, that the intake of a single dose of broccoli extract sprouts containing 200 µmol ITC, in which all glucosinolates had been hydrolyzed and the majority of the ITC were constituted by SF. ITC were absorbed rapidly and reached a peak plasma concentration of 0.94-2.27 µmol/l, 1 h after ingestion. The cumulative excretion of ITC equivalents at 8 h represented to 58.3 ± 2.8 % of the ingested dose (Ye et al. 2002). In a similar experiment the cumulative excretion of ITC equivalents after 72 h was 88.9 + / -55 % of the dose.

4.4 Bioavailability of dietary ITC

Understanding of factors, affecting the release of phytochemical components from the food matrix, their absorption and their fate in the body, is essential to determine the mechanism of action and biological effect.

There are several factors that can influence ITCs and GLSs : the plant variety, (Kushad et al. 1999; Mithen et al. 2001), agronomic and environmental factors such as soil and climatic conditions (Ciska et al. 2000; Pereira et al. 2002; Vallejo et al. 2003), but also plant storage and technological processes before consumption and cooking. Cold storage can cause the loss of nearly 50 % of GLSs, while cutting promotes the hydrolysis only on the cut surface. So in intact broad leaves or in broccoli or cauliflower inflorescences there is minimal GLS loss before cooking. If these vegetables are eaten raw, both intact GLS and active myrosinase are assumed simultaneouslyin the hydrolysis of GLS, occurs in the gastrointestinal tract. Some of the

GLS ingested are hydrolyzed in the colon by bacterial myrosinase, anyway myrosinase contained in the plant is the dominant factor.

Assuming that there is a large loss of GLS in the cooking water (between 30 and 60%), due to their hydrophilicity, it is evident that the preparation method can make a big difference on the assumption of GLS and on bioavailability of their hydrolysis products. An important factor in determining levels of ITCs intake is the combined influence of the cooking and the microbiota in the intestinal tract : mild cooking conditions (less than 75-80 ° C) cause an increased production of ITCs allowing the release of myrosinase, but the myrosinase rapidly denaturates when the temperature reaches 90 ° C . The content of ITC in the initial preparation was about the same (1.1 and 1.0 micromol /g wet weight), however, the urinary ITCs excretion in 24h was 32.3 % for raw broccoli and 10.25 % for cooked. These results indicate that ITCs bioavailability in raw vegetables, where the myrosinase had not been inactivated by heat, was three times higher (Conaway et al. 2000). Following administration of broccoli sprouts extract containing only ITC (after complete hydrolysis) or GST alone (in which myrosinase had been completely inactivated), the cumulative urinary excretion of ITC equivalents was to 80 % and 12 % of the dose respectively in 72h (Shapiro et al. 2001). These studies clearly show that plants in which myrosinase is still functional purchase a quantity of ITC amount much larger than those in which myrosinase is inactive. On the other hand different types of Cruciferous vegetables including broccoli and cabbage form nitriles (no anticarcinogenic properties known) as hydrolysis products

of GLS (Bones et al. 2006; Matusheski et al. 2006).These Cruciferous vegetables contain a ESP-like protein (ESP) that appears to be responsible for the formation of epithionitriles. ESP a not only catalyzes GLS hydrolisis, but also acts as a cofactor of myrosinase directing unstable products of hydrolysis to epithionitriles rather than to ITC. The ESP is more thermolabile than myrosinase and therefore a bland and short heat treatment can lead to a high ITC production, since this renders inactive ESP leaving a certain amount of active myrosinase (Bones et al. 2006). It is also clear that the myrosinase activity , employed by the intestinal microbiota, can hydrolyze only a small fraction of ingested GLS. Nevertheless when the microbiota is reduced by a combination of mechanical removal and antibiotic therapy, there is a further reduction in the ITC urinary excretion in from 11.3 ± 3.1 % to 1.3 ± 1.3 % of the dose.

4.5 Biological activities of sulforaphane

SF induces a phase II detoxification response promoting a disruption of nuclear factor E2-factor related factor (Nrf2)-Kelch-ECH-associated protein (Keap1) intereactions and mitogen-activated protein kinase activation. As a result, Nrf2 modulates gene expression via antioxidant response element (ARE). ARE-driven targets include NQO1, HO-1 and γ GCL and the induction of these enzymes has been observed both, “in vivo” and “in vitro” experiments after SF tratment. As we mentioned aboe, SF in contained in broccoli and this is the reason why several experiments have been done with dietary

ingenstion of this cruciferous vegetable. For example, dried broccoli sprouts (200mg/day) were able to attenuate oxidative stress, hypertension and inflammation in stroke-prone spontaneusly hyertensive rats (Wu et al. 2004). In addition, the antihypertensive effect of broccoli sprouts was accompanied by an enhancement in the GSH concentration and in the activities of GPx and GR in hearts, kidney, aorta and carotid, and by decreasing the macrofage infiltration in inner intimal layers of the aorta, carotid artery and endocardium of the heart, as well as in the kidney medullary interstitium and tubules.These beneficial effects were associated to the supression of the NF-kB pathway (Wu et al. 2004). Later, a cardioprotective effect was found after feeding broccoli for 30 days to rats in isolated heart preparations submitted to ischemia and reperfusion (Mukherjee et al. 2008). Rats feed with broccoli had an increased postischemic ventricular function and reduced myocardial infarct size along with reduced cardiomyocytes apoptosis. These protective effets were associated with a prevention in the decrease of thioredoxin, glutaredoxin and peroxiredoxin, HO-1, SOD1, SOD2 and Nrf2 as well as enhanced induction of the survival signalling proteins including Bcl-2, Akt, extracellular signal-regulated kinase ½, and down-regulation of the proteins (Bax, Jnk, p38) of the death signalling pathways.

4.6 Protective effects of SF

4.6.1 Brain and neuronal injury

Sulforaphane has a neuroprotective effects in several experimental paradigms. (Zhao et al. 2006) showed that a single SF administration was able to reduce the infarct size in rats induced by ischemia and reperfusion by increasing HO-1 espression in brain. It is important to noticethat HO-1 is an inducible enzymes that catabolizes free heme into carbon monoxide, iron and biliverdin, which is converted to bilirubin by biliverdin reductase. The same protective effect was observed in a neonatal hypoxia-schemia brain injury model (Ping et al. 2010) in which SF decreases malondialdehyde and 8-hydroxy-2-deoxyguanosine levels. In addition, a model of oxugen and glucose deprivation in immature neurons and in astrocytes (Danilov et al. 2009; Soane et al. 2010). SF protects the cells activating the Nrf2/ARE pathway, increases the gene trasciption protein levels and activity of antioxidant enzymes including NQO1, HO-1 and γGCL modifier subunit. Interestingly, SF also protects blood brain barrier after brain injury which was accompanied by the enhanced expression of Nrf2-driven genes (Zhao et al. 2007). SF njection protected against cerebral damage induced by instriatal injection of autologous blood (Zhao et al. 2007) and also activated Nrf2 brain tissue and reduced neutrophile count, oxidative damage, and behavioral deficits. Nrf2-deficient mice showed more severe neurologic deficits and did not benefit from the protective effect of SF. The above described protection was not observed in mice lacking by Nrf2 gene and

suggesting that this protection was dependent of Nrf2 (Zhao et al. 2007). In another brain experimental model, lipopolysaccharide-induced inflammation was attenuated by SF pre treatment with Nrf2 induction and HO-1 espression in the hippocampus of these animal brain (Innamorato et al. 2008). The role of Nrf2 in this protective effect was confirmed by using Nrf2 deficient mice. To add strength, "in vitro" studies have been done using SF and results also corroborate the protective SF effect and add more information about the mechanism of protection. For example, in BV2 microglial cells, the protective effect of SF against oxidative effect of lipopolysaccharide was associated with HO-1 induction (Innamorato et al. 2008). In another cell culture the dopaminergic cell death, induced by a compound that produces dopamine quinone: 6-hydroxydopamine and tetrahydrobiopterin, was also attenuated by SF preincubation (Han et al. 2007). These experiment demonstrated that SF pretreatment prevented membrane damage, DNA fragmentation, and ROS formation. SF, increased mRNA levels and enxymatic activity of NQO1 in a dose-dependent manner (Han et al. 2007). In another experiment model, SF protects cortical neurons against the neurotoxin 5-5-cysteildopamine damage, because SF induces the expression and the activation of Nrf2 and therefore increases the espression and activity of GST, GR and NQO1 (Vauzour et al. 2010). Interestingly, SF increases in time-concentration manner reduced GSH levels in dopaminergic-like neuroblastma SHSY-5Y cell line which is associated with its protective effect againsy hydrogen peroxide (Tarozzi et al. 2009).

4.6.2 Liver damage

Baek et al.(2008) demostrated the protective effect of SF on carbon tetrachloride-induced liver injury in mice. They showed that SF ameliorated the carbon tetrachloride induced increase in the serum level of alanine amnotransferase, lipid peroxidation and necrosis. This hepatoprotective effect was associated with liver phase II enymes induction. In this sense Li iet al. (2010) have shown that SF upregulated the expression of the π class of GST through the Nrf2 pathway in rat Clone 9 liver celle. Moreover Razis et al. (2010) also demonstrated that R-SF is more effective a an inducer of the detoxifying enzymes system, in bothliver and lung, compared with the S-enatiomer. Finally Zhao et al.(2010) is investigated the effect of SF on regulation of Nrf2/ARE pathway in liver injury induced by intestinal ischemia/reperfusion. They showed that the pretreatment with SF ameliorates the ischemia-reperfusion induced intestinal and liver injury. This protection was associated to the increase in liver expression of Nrf2 and HO-1.

4.6.4 Hyperglycemia

Xue et al.(2009) demonstrated that treatment with SF in a human microvascualar HMEC-1 endothelial cells model incubated in lowand hignt glucose concentration can prevent ROS production, mitochondrial and biochemical dysfunction induced by hyperglycemia which was associated to increased expression of transketolase. Song et al.(2009) have found that SF pretreatment was able to ameliorate

streptozotocin-induced islet damage in mice. SF pretreatment was able to ameliorate hyperglycemia and decreased insulin blood concentration and to preserve the number of islets producing insulin. The protective effect of SF in streptozotocin induced diabetes was associated to the prevention of the induction of NF-kB in islets of thee mice. Similar "in vitro" results were presented in rat pancreatic β-cell line RINm5F, where SF preincubation was able to induce Nrf2 traslocation into the nucleus and subsequnce gene expression of several cytoprotective enzymes including γGCL (Song et al. 2009). In addition, preincubated pancreatic β cells with SF are resistant to the toxic effect of citokynes preventing H_2O_2 formation. SF also suppressed the increae in inducibile nitric oxide synthase induction and cyclooxygenase expression (Song et al. 2009).

4.6.5 Damage to heart and cardiac cells

Zhu et al.(2008) showed that the incubation of rat aortic smooth muscle A10 cells with different concentrations of SF induced in concentration –dependent manner the level and activity of antioxidant and phase II enzymes such as catalase, SOD, GPx, GR, GST, NQO1 and GSH. SF can also induce the expression and the activity of catalase, GSH, SOD and GST in isolated mitochondria of aortic smooth muscle cells. In the same study, the pretreatment of SF prevented the cell death, ROS production and oxidative cytotoxicity by xanthine oxidase/xanthine and H_2O_2. It's has been demonstrated

that SF increased the gene expression, protein expression and enzyme activity of phase II enzymes including GR, GST, NQO1, TRr in cultured rat neonatal cardiomyocytes model (Angeloni et al. 2009). These increases were in time-concentration manner. SF pretreatment was able to avoid the cell death, ROS production and DNA fragmentation induced by H_2O_2 in cardiomyocytes. On the other hand, SF prevented the ischemia-repurfusion injury in hearts (Piao et al. 2009). The protective effect was observed by an inhibition of the increase in the post-ischemic left ventricular end-diastolic pressure and improved the post-ischemic left ventricular developed pressure coronary flow reduction in the infracted area and decreased lactate dehydrogenase levels during reperfusion. SF prevented the decrease in protein expression of some antioxidants enzymes including catalase, Mn-SOD and HO-1.

CHAPTER 5

5. Aim

Chronic degenerative diseases represent the main cause of mortality in the industrialized world. A common determiner of these pathology is oxidative stress, a great imbalance between reactive oxygen species and antioxidant defences with resulting damages to biological macromolecules. In the last years, many studies have directed their attention to carbonyl stress, a condition wherein there is an imbalance between pro-glycant species production and organism defences to glycation. Carbonyl stress is probably responsible of Alzheimer disease, renal dysfunctions and cardiovascular diseases. It was observed that after ischemic stroke there is an increase of oxidative stress, to which are associated several troubles. It's very important to remember that the antioxidant enzymes activity in the heart is lesser than other organ. For this reason the heart is more susceptible to oxidative stress. Therefore, any mechanisms, able to increase antioxidant defences, result fundamental to protect cardiac cells from damages induced by oxidative stress. In 1986 Murry et al. (Murry et al. 1986) described ischemic preconditioning (PC) as a phenomenon whereby myocardium exposed to brief episodes of ischemia and reperfusion develops protection against irreversible injury during a

subsequent ischemic insult. This phenomenon has been recognized as the strongest form of in vivo protection against myocardial ischemic injury (Kloner et al. 1998). In experimental animals, a brief period of ischemia usually produces two windows of protection: an early phase which develops very quickly and lasts only 1-2 h, and a late phase that develops after 12-24 h but lasts 3-4 days (Kloner et al. 2001; Eisen et al. 2004). The early phase develops by rapid post-translational modification of pre-existing protein through a series of signaling cascades, while late (or delayed) ischemic PC is mediated by cardioprotective gene expression and by synthesis of new cardioprotective proteins (Rizvi et al. 1999; Das et al. 2008).

The ischemic PC concept has also been extended to PC triggered by non-ischemic stress, such as stretch, some chemicals (Shattock et al. 1993), and reactive oxygen species (ROS) (Leon et al. 1998). Redox signaling in PC is still not completely understood, but it is widely accepted that transient, low concentrations of ROS and/or reactive nitrogen species (RNS) may trigger protective mechanisms. Some ROS (i.e. O_2^- and H_2O_2) and RNS (i.e. NO, HNO, $ONOO^-$) may be included among the triggers of PC, and it is likely that they collaborate in inducing cardioprotection (Penna et al. 2009). Recently, the role of H_2O_2 as PC inducer in the protection against different forms of damage has received attention. Sharma et al. (2001) have observed that H_2O_2 induced PC may provide cardioprotection, similar to ischemic PC, against ischemia–reperfusion injury in isolated rat heart. In a mouse L-cell model, H_2O_2 PC protected cells against apoptosis induced by subsequent oxidative stress via MAPK and

PI3K/Akt pathways (Han et al. 2001). In addition, H_2O_2 PC has been shown to protect human proximal tubular cells against lethal oxidant insult via p38 MAPK and heme oxygenase-1 (Lee et al. 2003). In more recent studies, it has been demonstrated that H_2O_2 PC protects PC12 cells against apoptosis induced by oxidative stress through different mechanisms: blockade of reductions in mitochondrial membrane potential, overexpression of Bcl-2 (Tang et al. 2005) and inducible nitric oxide synthase and cyclo-oxygenase-2 (Tang et al. 2006), and activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Yu et al. 2006), and increase in the DNA binding activity of NF-kB p65, as well as its nuclear translocation (Zhang et al. 2009).

Another strategy to protect cardiac cells from oxidative damage is an up-regulation by natural or synthetic chemical inducers, of endogenous antioxidants and phase II enzymes of xenobiotic metabolism (Cao et al. 2006). The main effect of this induction would be a reinforcement of antioxidant cellular capacity. Among them, sulforaphane is one of the most promising diet-derived indirect antioxidant agents. SF is produced by the breakdown of glucoraphanin, a glucosinolate abundantly present in some Cruciferous vegetables, especially broccoli. It has been reported that broccoli protects hearts against I/R injury through the redox cycling of the thioredoxin superfamily (Mukherjee et al. 2008). We have demonstrated that SF protects cardiomyocytes against apoptosis induced by oxidative stress (Angeloni et al. 2009). In a recent study we have also identified that SF modulate other molecular targets such

as GLO1. GLO1, GLO2 enzymes and a catalytic quote of reduced GSH form the glyoxalase system. This system catalyze the conversion of α-ossoaldeidi in α-idrossiacidi. This allow the biotransformation of toxic compound for the cell, such as methylglyoxal, in no toxic compound, D-lactate. Glyoxalase system represent a detoxifying system to dicarbonyl species, and so, as an integral part of organism antioxidant defences. This is also fundamental to limit the toxicity induced by carbonyl stress, apoptosis and AGEs formation. AGEs are an heterogeneous group of molecules that are generated through a non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids (Thorpe et al. 2003). A great presence of AGEs at cellular level, is related with Alzheimer ,Parkinson and cardiovascular disease. Previous studies have been shown that the glycation generate the AGEs production on β-amyloid plaque (Chen et al. 2006), that MG is the main glycant agent in this process (Webster et al. 2005). These observations have suggested that AGEs can promote the depositing of β-amyloid and plaques in brain with AD. The glyoxalase system was related with chronic pathology induced by oxidative stress or hyperglycemias. In particular a decrease of GLO1enzymtic activity in situ caused by ageing and oxidative stress increase the glycation. The oxidative stress is bound glycation because the depletion of GSH cause a decrease of GLO1 enzymatic activity and an increase of methylglyoxal and glyoxal.

The aim of this thesis was evaluated molecular mechanisms in cardio- and neuroprotection and the possibility of modulation by nutraceutical phytocomponents.

CHAPTER 6

6. Materials and Methods

6.1 Materials

PhosSTOP was purchased from Roche Diagnostics (Mannheim, Germany). CelLytic M, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, fetal bovine serum (FBS) , mammalian protease inhibitor mixture, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, bovine serum albumin, NADP, dimethyl sulfoxide (DMSO), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glutamine, penicillin-streptomycin, reduced glutathione (GSH), 2 LY 294002 (LY), PD 98059 (PD), SB 203580 (SB), DMEM F12, DMEM, fetal caw serum (FCS), horse serum (HS), Gentamicin, Amphotericin B, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), 7-amino-4-Methylcoumarin (AMC), trypsin, and all other chemicals of the highest analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,8-phenanthridinediamine, 5-(60-triphenylphosphoniumhexyl)-5,6 dihydro-6-phenyl (MitoSOX) was purchased from Invitrogen (Paisley,

UK). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR,USA).

6.2 Cells line

Neonatal Wistar rat cardiomyocytes were isolated as reported (Hrelia et al. 2002). Briefly, cells were obtained by isolation of cardiomyocytes from the ventricles of 2–4 days old Wistar rats, seeded at a density of $1x10^5$ cells/cm^2 and grown until confluence in DMEM F12 supplemented with 10% v/v FCS, 10% v/v HS, 1% v/v gentamicin, 1% v/v Amphotericin B and 1% v/v sodium pyruvate and incubated at 37C° in 5% CO2 humidified atmosphere.

The human neuroblastoma cell line SHSY-5Y was purchased from Sigma Chemical Co. (St.Louis, MO, USA). The cells were grown in DMEM medium supplemented with 10% v/v heat inactivated FBS, 1% v/v 200mM gentamicin and 1% v/v penicillin-streptomycin.

6.3 Preconditioning, oxidative stress, carbonyl stress and SF treatment

PC was simulated with different concentration of H_2O_2 (1µM, 5µM, 10µM, 50µM and 100µM) for 10 minutes.

Oxidative stress was induced by exposing the cells to H_2O_2 100µM for 30 minutes.

Carbonyl stress was induced by exposing cardiomyocytes to MG 1mM for 24 hours and SHSY-5Y to MG 0.5mM for 24 hours

Cells were treated with 5 μM SF for different time points, and control cells were treated with equivalent concentrations of DMSO alone. SF concentration utilized in this study is readily achievable in rat and human plasma (Hu et al. 2004; Gasper et al. 2005).

6.4 Cell viability assay

Cell viability was evaluated by measuring MTT reduction. At the end of each experiments, MTT was added to the cell medium (final concentration 0.5 mg/mL) and incubated for 1 h at 37° C. After incubation, MTT solutions were removed, DMSO was added and the absorbance was measured using a microplate spectrophotometer (VICTOR3 V™ Multilabel Counter, Perkin Elmer e Wellesley, MA, USA) at a wavelength of 595 nm.

6.5 Caspase-3 activity assay

The activity of caspase 3 was measured by hydrolysis of the peptide substrate Ac-DEVDAMC by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin moiety (Nicholson et al. 1995). Cells were lysated in lysis buffer (50 mM Tris, 0.1% Triton X-100, 150mM NaCl, 2mM EGTA/EDTA, 1mM sodium pyrophosphate, 10 mg/mL phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mg/mL aprotinin and then centrifuged 5 min at 5000 g. From each sample, 5μl was transferred to a 96-well plate and 200μl of reaction mix (20 mM HEPES pH 7.4, 0.1% CHAPS, 5mM DTT and 2mM EDTA) containing Ac-DEVDAMC (final

concentration 16 μM), was added to each well. Fluorescence intensity was recorded every 5 min for 1 h by amicroplate spectrofluorometer (λex/em= 360/460 nm). Caspase activity was calculated using a AMC standard curve and results were expressed as nmol AMC/min/mg protein.

6.6 Detection of intracellular ROS production

- **Confocal microscopy:** ROS formation in cardiomyocytes was visualized as follows. Cells were incubated with 5 μM SF for 24 h, exposed to1 mM MG for 24 h, and then washed twice with warm PBS, and DHE and MitoSOX were added to cells at a final concentration of 5 μM. After incubation with DHE (20min) and MitoSOX (10 min), cells were fixed with 4% paraformaldehyde for 10 min, mounted onto glass slides with Mowiol, and observed under a confocal microscope with excitation and emission wavelengths set to 490 and 590 nm,respectively (Marella et al. 2007; White et al. 2008). All images were taken under identical exposure conditions in order to evaluate the intensity of the probe fluorescence accurately. Confocal imaging was performed on a Nikon A1 confocal laser scanning microscope as previously described (Resca et al. 2013). The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections, as previously described (Maraldi et al. 2013). The image rendering was performed by Adobe Photoshop software.

- **Spectrofluorimetric assays:** ROS production was evaluated using different fluorescence probe: DCFH-DA, DHE and MitoSOX. At the end of treatment cells were washed with PBS and then incubated with 5 μM DCFH-DA for 30 min. Next, cells were incubated with 1mM MG. Cell fluorescence was measured using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter) (λex/em=485/535nm) as previously reported (Bradford 1976). Cells were treated with 5 μM SF for 24 h and then exposed to 1 mM MG. Cells were washed with PBS and incubated with 5 μM DHE for 20 min or 5μM MitoSOX for 10 min at 37 °C. Cell fluorescence was measured using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter) (λex/em=485/590nm).

- **Total antioxidant activity (TAA):** TAA assay, performed as previously reported (Bordoni et al. 2005), was used as an indirect index of intracellular ROS production. Briefly, at the end of each experiment, cardiomyocytes were washed 3 times with cold PBS. Cells were lysated in PBS using a potter homogeniser and centrifuged at 1000g to remove cell debri. TAA was determined by the decoloration of the radical cation ABTS, in terms of quenching of absorbance at 740nm. Values obtained for each sample were compared with the concentration– response curve of a standard Trolox solution, and espresse as μmol of Trolox Equivalent Antioxidant Activity per mg of protein (TEAA μmol/mg protein).

6.8 Western blotting

Preparation of nuclear and cytoplasmic fractions were performed according to the method of (Bahia et al. 2008) and whole-cell extracts with CelLytic ^{TM}M Cell lysis reagent, with mammalian protease inhibitor mixture and PhosSTOP. Samples were boiled for 5 min prior to separation on 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) in Tris-glycine buffer at 110 V for 90 min. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-GST (Alpha Diagnostic International, San Antonio, TX, USA), anti-GR (AbFrontier, Seoul, Korea), anti-TRred (Upstate, Lake Placid, NY, USA), anti-GPX1 (Lab Frontier, Seoul, Korea), anti-NQO1, anti-SOD1, anti-SOD2, anti-CAT, anti-Nrf1, anti-Nrf2, anti-GLO1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-p38, anti-phsopho-ERK1/2, anti-phosho-Akt, anti-Bcl2, anti-Caspase 3 (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (SIGMA) and anti-Histone H3 (Cell Signaling Technology), as internal normalizers, overnight at 4°C on a three-dimensional rocking table. The results were visualized by chemiluminescence using ECL® Advance reagent

according to the manufacturer's protocol (GE Healthcare). Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer (Bio-Rad, Hercules, CA, USA).

6.9 Enzymatic activity assays

NAD(P)H quinone oxidoreductase 1 (NQO1) enzymatic activity was measured according to the procedure of Prochaska et al. (1988). Glutathione S-transferase (GST) activity was assayed according to the procedure of Habing et al. (1974). Thioredoxin reductase (TRred) activity was assayed according to the procedure of Holmgren and Bjornstedt (1995). Glutathione reductase (GR) activity was measured according to the method of Smith et al. (1988). Glutathione peroxidase (GPX) activity was assayed according to the method described by Flohe et al. (1984). Catalase (CAT) activity was determined according to the method of Johanssonet al. (1988). Superoxide dismutase (SOD) activity was measured according to the method of Peskin et al.(2000). Glyoxalase 1 (GLO1) activity was measured according to the method of Mclellan anf Thornalley (1989).

6.10 AGE assay

AGE protein adducts were quantified by the OxiSelect™ AGE ELISA Kit (Cell Biolabs, San Diego, CA, USA) according to manufacturers' instructions

6.11Detection of reduce GSH level

Reduce GSH level was evaluated using a thyole reactive dye monochlorobimane (MCB) which is essentially non-fluorescent until it reacts with GSH to form a fluorescent GSH-MCB conjugate (Rice et al. 1986). At the end of treatments, cells were incubated with 50μM MCB in HBSS for 30 minutes. Cell fluorescence was measured using 355 excitation and 460nm emission with a microplate spectrofluorometer (VICTOR 3 V^{TM} Multilabel Counter)

6.12 Small-interfering RNA transfection

Cardiomyocyteswere transfected with 50 nM Nrf1- and Nrf2-annealed small interfering (si)RNA (Invitrogen, Pisley, UK) using Lipifectamine 2000 for 12 h according to manufacturer's recommendations. The siRNA sequences utilized targeted the following rat Nrf1 coding sequences: 5'-GACUUCUUGGACAAGCAGAUGA-3' and 5'-UCAUCUGCUUGUCCAAGAAGUCAGC-3' and the following rat Nrf2 sequences: 5'-UGGAGCAAGACUUGGGCCACUUAAA-3' and 5'-UUUAAGUGGCCCAAGUCUUGCUCCA-3'. Control expriments were formed using equivalent amounts of the StealthTM RNAi Negative Control Med GC (Invitrogen)..

6.13 Protein concentration

The protein concentration of the cell lysates was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

6.14 Statistics

Each experiment was performed at least three times, and all values are represented as means ± SD. One-way analysis of variance (ANOVA) was used to compare differences among groups followed by Dunnett's or Bonferroni's test (Prism 5, GraphPad Software Inc., San Diego, CA, USA). Values of $p<0.05$ were considered as statistically significant.

CHAPTER 7

7 RESULTS

7.1 Protective effects of H_2O_2 preconditioning Against oxidative stress in cardiomyocytes

7.1.1 Preconditioning protective effect

First objective of this thesis was to determine the concentrations of H_2O_2 able to simulate ischemic preconditioning (PC) in vitro. Cardiomyocytes were treated with different concentrations of H_2O_2 (1, 5, 10, 50, 100 µM) for 10 min (preconditioning) and after 24 h were subjected to oxidative stress induced by hydrogen peroxide 100 µM for 30 min. Cell viability was assessed by MTT assay 24 h after oxidative stress.

In cells not preconditioned, oxidative stress determined a significant reduction of cell viability of about 50 % (Fig.7-1). Treatments with 1-10 µM H_2O_2 did not show any protective effects against oxidative stress, meanwhile 50 and 100 µM H_2O_2 were able to significant increase cell viability in respect to cells exposed to oxidative stress. Interestingy, treatment with 100 µM H_2O_2 increased cell viability to values comparable to control cells.

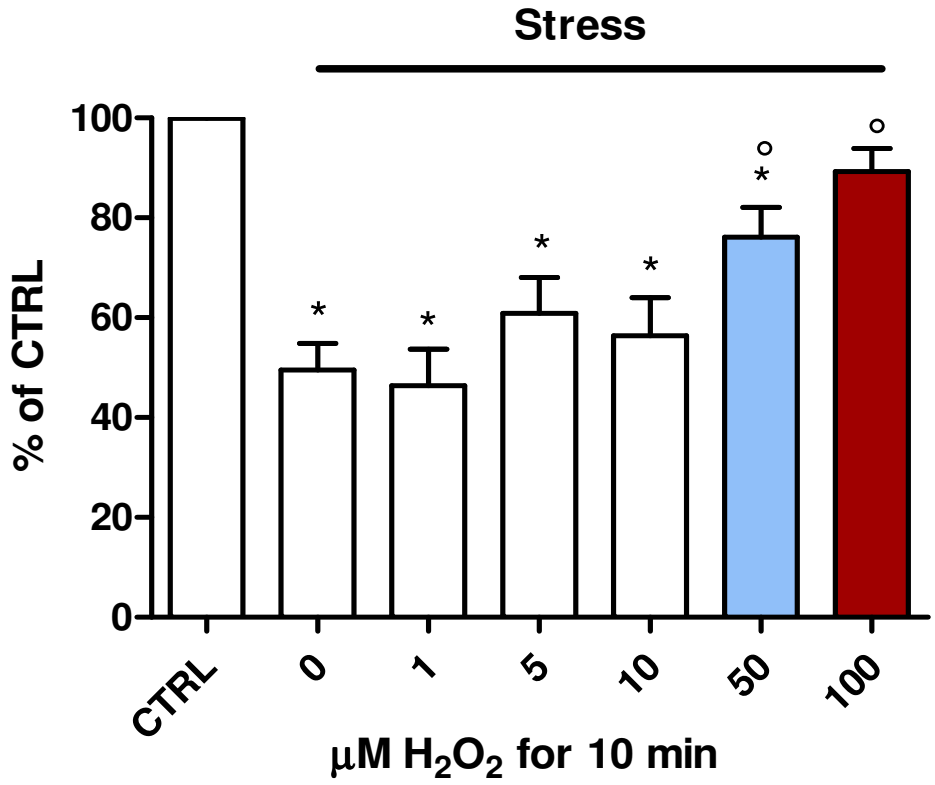


Figure 7-1: Effect of H2O2 preconditioning (PC) on cell viability in cardiomyocytes exposed to oxidative stress. *Cells were treated with increasing concentrations of H_2O_2 (1-100 µM) for 10 min. After 24 h, cells were stressed with 100 µM H_2O_2 for 30 min and cell viability was assessed by the MTT assay and reported as %cell viability compared with controls .Each bar represents means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control; °p< 0.05 vs 0 (Stressed cells)*

Ischemic PC is a phenomenon that has a biphasic pattern characterized by two different time windows of protection: a first phase of protection (or first window of protection) that develops immediately and disappears within 1-2 hours after the induction of PC (VanWinkle 1991) and a second phase of protection (or second window of protection) that occurs 12 hours later by the induction of the PC and lasts for 72-96 hours (Marber et al. 1993). To understand if the main contribute of H_2O_2 PC is in the early or delayed PC, cells were preconditioned with H_2O_2 and stressed after different time from the induction of PC (1–72 h). Data reported in figure 7-2 show that after 1 and 2 h from PC cell viability was significantly higher than that measured in not preconditioned cells. After 24 h from PC, cells are able to counteract oxidative stress damage with the highest efficiency maintaining cell viability at value comparable to control cells.After 48 and 72 h from PC, H_2O_2 is still able to counteract oxidative stress injury but to a lesser extent than after 24 h from PC. These results suggest that H_2O_2 PC exerts its cardioprotectivea ction mainly in the late phase of PC.

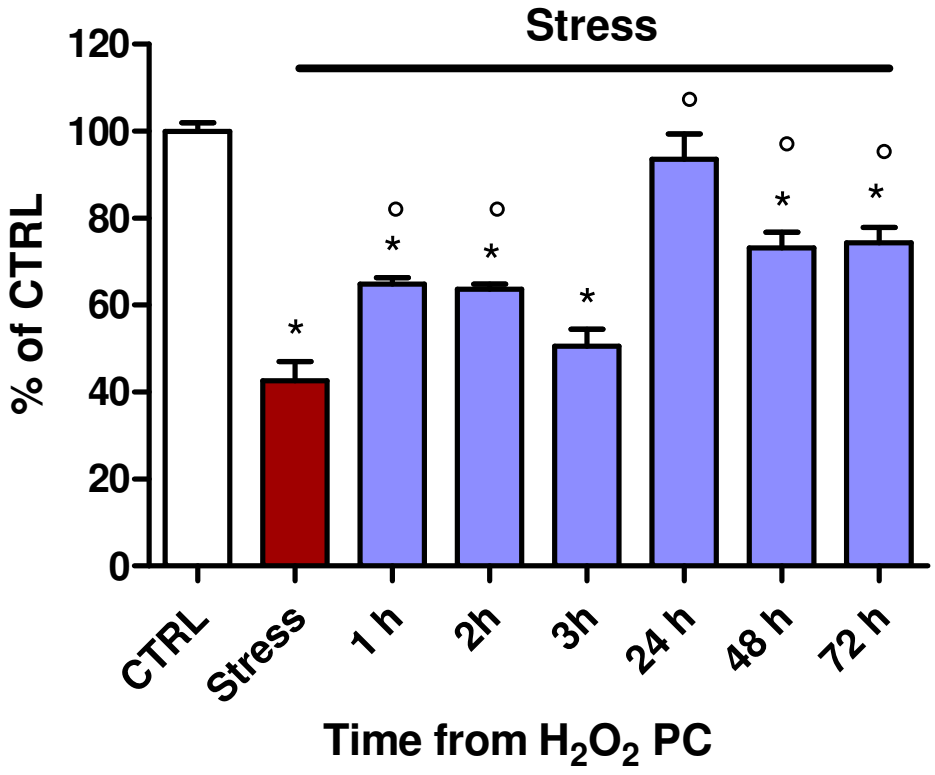


Figure 7-2: Cell viability of cardiomyocytes subjected to H_2O_2 PC and oxidative stress at different times. *Cells were treated with 100 µM H_2O_2 for 10 minutes and recovered for different times periods (1-72 h) before the induction of oxidative stress. Cell viability was assessed by the MTT assay and reported as % cell viability compared with controls. Each bar represent means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs 0 (Stressed cells)*

To better understand the role of H_2O_2-PC against oxidative stress, we evaluated its effect against apoptosis. Apoptosis was assessed by flow cytometry, caspase-3 activity and Bcl2 protein levels (Fig.7-3).

Cell were preconditioned with H_2O_2 100 µM and after 24 h were exposed to oxidative stress.

Cytofluorimetric assay (figure 7-3 A) revealed that H_2O_2-PC protected cardiomyocytes against apoptosis induced by oxidative stress. H_2O_2-PC alone did not influence the percentage of apoptotic/necrotic cells in respect to controls, while oxidative stress induced a marked shift of the population from viable cells to early and late apoptotic cells. Preconditioning before oxidative stress was able to reduce the shift of cells from the viable to the apoptotic region in respect to oxidative stress alone.

To further demonstrate the protection of 100 µM H_2O_2-PC against apoptosis, the activity of caspase-3, a key enzyme required for the execution of apoptosis, was measured. The activation of this enzyme represents a consolidated biomarker of apoptotic death. Cell were preconditioned with 100 µM H_2O_2 and after 24 h were exposed to oxidative stress (Fig.7-3 B). PC alone had no significant effect on caspase-3 activation. Oxidative stress significantly increased caspase-3 activity in cardiomyocytes, while H_2O_2-PC significantly reduced caspase-3 activity induced by oxidative stress.

One of the major genes responsible for regulating apoptosis is the proto-oncogene Bcl-2 localized to the nuclear membrane, endoplasmic reticulum and the outer mitochondrial membrane. It has been

demonstrated that Bcl-2 protein plays an anti-apoptotic role in cardiac cells. We evaluated the expression of Bcl2 in preconditioned cardiomyoctytes after different time points from PC (figure 7-3 C). Immediately after H2O2 PC induction, Bcl2 expression was significantly increased compared with control cells and this induction lasted until 24 h from H2O2 PC. At 48 and 72 h from PC, Bcl2 expression was comparable to control cells.

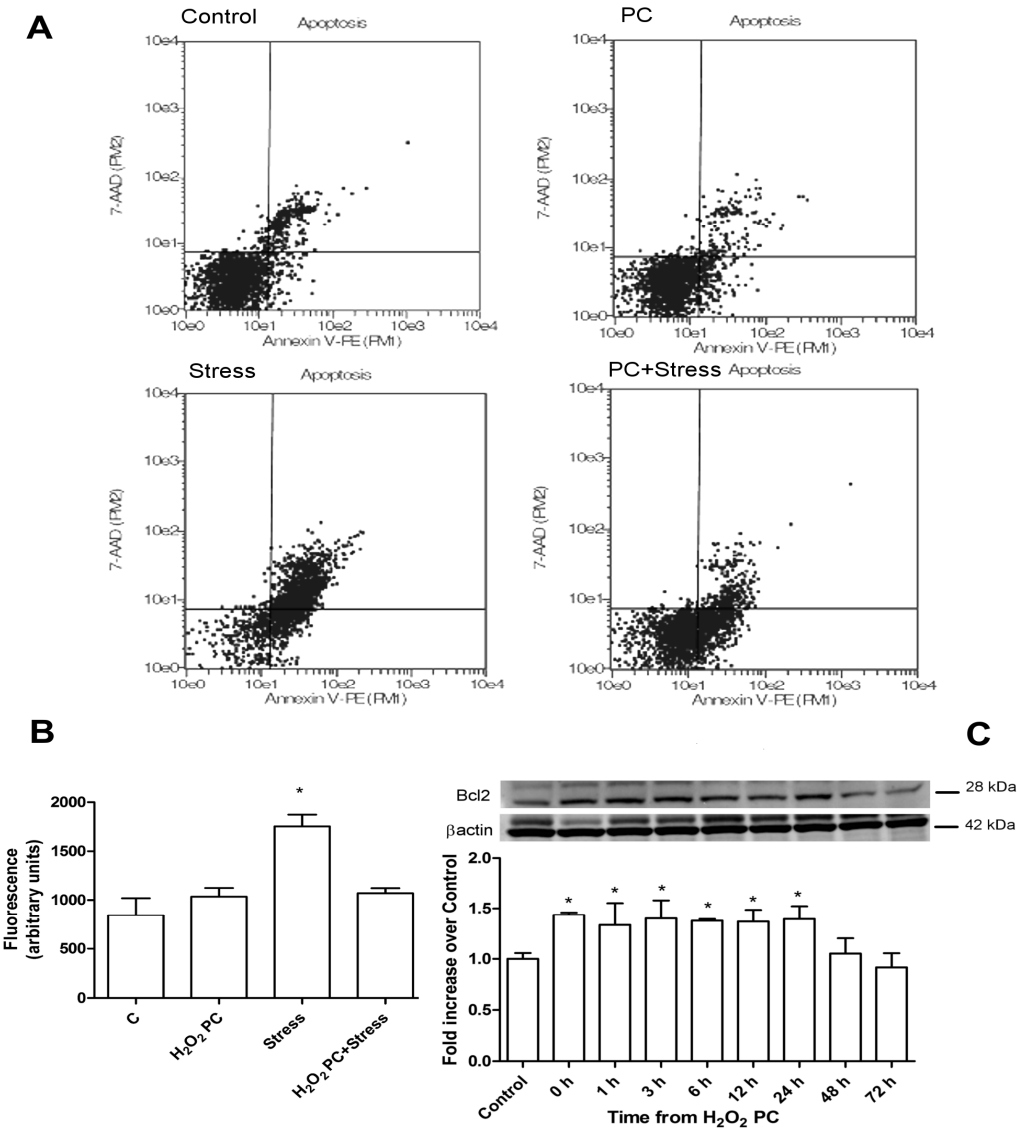


Figure 7-3: ***Effect of H_2O_2-PC on apoptosis.*** *Apoptosis was assessed by flow cytometry (A), caspase-3 activity (B), and Bcl2 protein levels (C). A: cardiomyocytes were preconditioned with 100 μM H_2O_2 for 10 min and after 24 h were exposed to oxidative stress and flow cytometry was assesd using annexin V-phycoerythrin (PE)/7-amino-actinomycin D (7-AAD) described in the Materials and Methods chapter. B: caspase-3 activity was measured spectrofluorimetrically in cell lysates as reported in METHODS. Each column represents the means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *P _ 0.05, with respect to control; °P _ 0.05, with respect to stress. Cardiomyocytes were exposed to H_2O_2 100 μM for 10 minutes and lysates were obtained at different times after induction of the PC. Proteins were subjected to SDS-PAGE electrophoresis,s separated on the gel and transferred to nitrocellulose, incubated with anti-Bcl2 antibody and detected by chemiluminescence as described in the Materials and Methods chapter. Anti β-actin were used as controls charcing. Densitometric analysis was performed on three separate nitrocellulose membranes. The data are reported as an increase compared to the control ± SD. The statistical analysis was performed with the test one-way ANOVA followed by Dunnet test: *p< 0.05 vs Control cells*

7.1.4 Effect of H_2O_2 PC on intracellular ROS production

Intracellular ROS production was measured to study the involvement of PC in modifying intracellular redox state. Cardiomyocytes were treated with different concentrations of H_2O_2 and after 24 h were exposed to oxidative stress (Fig.7-4). Treatment with 1-10 µM H_2O_2 did not influence ROS levels compared to cells not treated and exposed to oxidative stress (control+). On the contrary, 50 and 100 µM H_2O_2 were able to significantly reduce ROS production compared to control+, in agreement with the data obtained with the MTT assay.

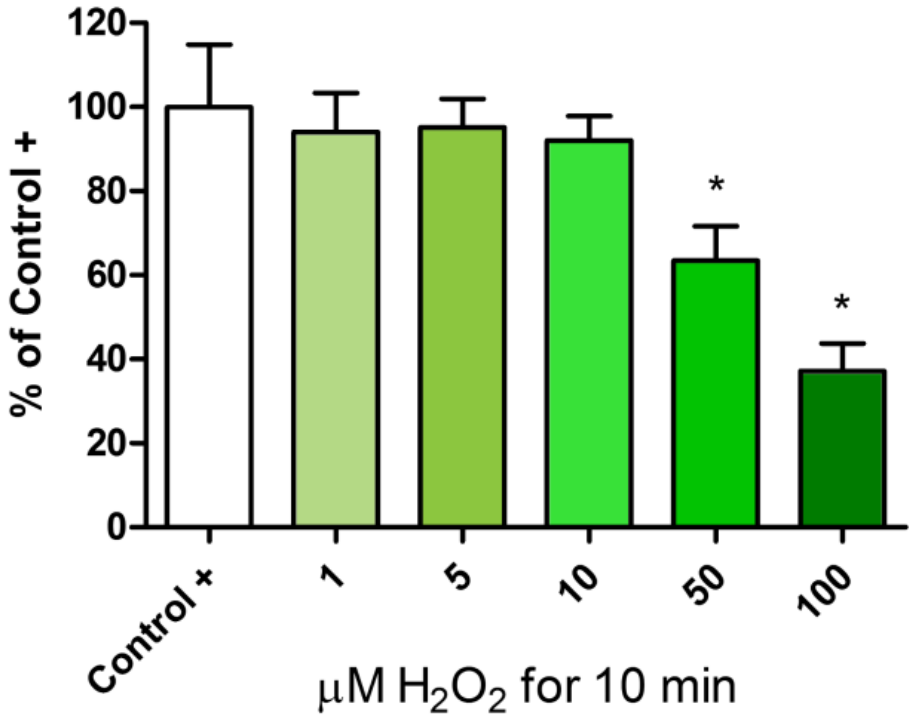


Figure 7-4: Preconditiong effect on the intracellular ROS production. *Cells were preconditioned with 1-100 μM H_2O_2 and after 24 h recovery were exposed to oxidative stress. Iintracellular reactive oxygen species was assessed by DCFH-DA assay and values represent means ± SD of 6 independent experiments. Data were analyzed by one-way ANOVA followed by Dunnet test: *p< 0.05 vs Control+*

7.1.5 Effect of H_2O_2 PC on antioxidant/phase II enzyme induction and activity

As H_2O_2 can not act as an antioxidant and its major protective effect is after 24 h from PC, we assumed that H_2O_2 PC antioxidant capacity could be ascribed to its ability to enhance the endogenous antioxidant defense system through the synthesis of new cardioprotective proteins. We therefore analyzed protein expression and activities of the main antioxidant and phase II enzymes: glutathione reductase (GR), thioredoxin reductase (TR), NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), glutathione peroxidase (GPX),catalase (CAT) and superoxide dismutase (SOD).

Cardiomyocytes were preconditioned with different concentrations of H_2O_2 (1-100 μM) for 10 minutes and protein expression was evaluated after 24 h by immunoblotting.

SOD1, SOD2, GPX, and GST expressions were not influenced by H2O2 PC at any tested concentrations (data not shown). On the other hand, GR, NQO1, and TRred expressions were significantly increased by 50 and 100 μM H2O2 PC, while CAT expression revealed a significant increase only at the highest H_2O_2 concentration (Fig.7-5).

1-10 μM H_2O_2 did not modify protein level in respect to controls . In contrast, the concentration of 50 μM induces significantly GR, TR and NQO1, while the concentration 100 μM was able to induce , in addition to GR, TR and NQO1 also CAT, GST, GPX, while SOD was not affected PC.

To evaluate whether protein expression modifications were related to functional effects, enzyme activities were measured in the same experimental conditions. SOD, GPX, and GST activities were not modulated by H_2O_2 PC in respect to control cells (Table 7.1). In agreement with expression data, GR, NQO1, and TRred activities were significantly increased by 50 and 100 μM H_2O_2 treatment, while CAT activity was increased only by100 μM H_2O_2 PC.

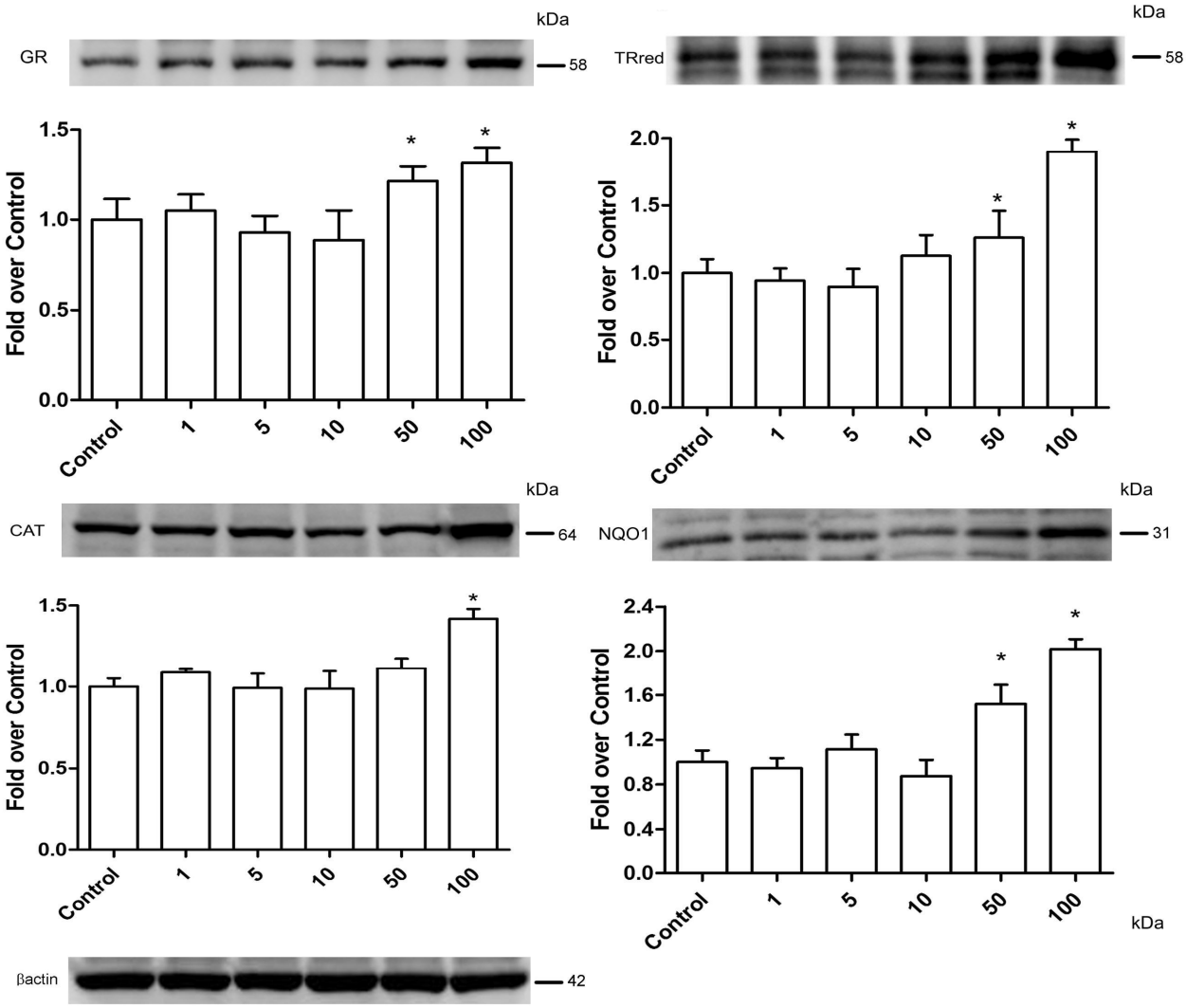


Figure 7-5: Preconditiong effect on the induction of antioxidant phase II enzymes. *Cardiomyocytes were preconditioned with H_2O_2 1-100 μM for 10 minutes. Cell lysates (20μg) were immunoblotted with antibodies that detect endogenous levels of glutathione reductase (GR), catalase (CAT), thioredoxine reductase (TRred), and NAD(P)H quinone oxidoreductase 1 (NQO1). Results of scanning densitometry analysis permorfed on 3 independent antoradiograph are presented. Relative amounts (means ± SD) were normalized to the intensity of the same β-actin blot and represented as fold increase in respect to control. Data were analyzed by one-way ANOVA followed by Dunnet's test: *p< 0.05 vs Control cells*

Table 7.1*: Effects of different concentrations of H_2O_2 for 10 min on SOD, GPX, GST, GR, TRred, CAT, and NQO1 activities*.

μM H_2O_2	SOD (U/mg protein)	GPX (mU/mg protein)	GST (nmol/min /mg protein)	GR (mU/mg protein)	TRred (mU/mg protein)	CAT (nmol/min/ mg protein)	NQO1 (noml/min/ mg protein)
0	2.20±0.17	48.70±3.06	11.29±0.68	10.92±1.18	52.72±1.22	39.12±2.73	10.17±1.54
1	2.23±0.16	54.98±3.43	10.96±0.64	10.98±0.47	53.47±5.98	41.93±4.80	10.30±1.30
5	2.22±0.19	54.80±5.72	11.02±0.47	11.40±0.68	50.46±2.73	40.17±3.75	9.86±2.42
10	2.30±0.14	53.13±10.11	10.03±0.54	10.43±0.26	53.84±6.07	37.17±5.44	12.99±3.98
50	2.33±0.16	51.06±5.00	11.07±1.11	14.19±0.78*	60.65±2.83*	43.37±1.88	18.16±2.85*
100	2.35±0.05	53.58±1.43	11.89±0.63	14.48±0.36*	76.77±5.94*	63.87±5.54*	20.38±2.85*

**Value represents the mean ± SD of four independent experiments. Cardiomyocytes were preconditioned with 1-100 μM H_2O_2 for 10 min, and after 24 h cells were lysed for enzymatic activity measures. GPX, glutathione peroxidase; GST, glutathione S-transferase; GR, glutathione reductase; TRred, thioredoxine reductase; CAT, catalase; NQO1, NAD(P)H quinone oxidoreductase 1. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. * $p<0.05$ with respect to 0 μM H_2O_2 (Control)*

7.1.6 Effect of H_2O_2 PC on Nrf1 and Nrf2 activation.

It has been shown that the expression of antioxidants and phase II enzymes is modulated by Nrf1 and Nrf2 thorugh the ARE/keap1 pathway (Brigelius-Flohe et al. 2006; Purdom-Dickinson et al. 2007; Zhu et al. 2008) . Therefore, we evaluated the effect of PC on Nrf1

and Nrf2 nuclear traslocation after different times from PC induction by analyzing both cytosolic and nuclear fractions (Figure 7-6 and 7-7). Results showed that PC induces Nrf2 translocation from the cytosol to the nucleus (Fig. 7-6). Indeed, Nrf2 expression in the nuclear fraction after 2 h from PC is significantly higher than that observed in control cells. In contrast, Nrf2 protein level in the cytosolic fraction decreases significantly after 0-3 h after PC, reaching levels similar or higher than those observed in control cells 6-24 h after PC induction.

In a similar way, Nrf1 is translocated from the cytosol to the nucleus following PC (Figure 7-7). Nrf1 protein level in the nuclear fraction is significantly higher than that observed in control cells already after 1 h after PC, while Nrf1 protein level in the cytosolic fraction decreases slightly after 2 h from the PC.

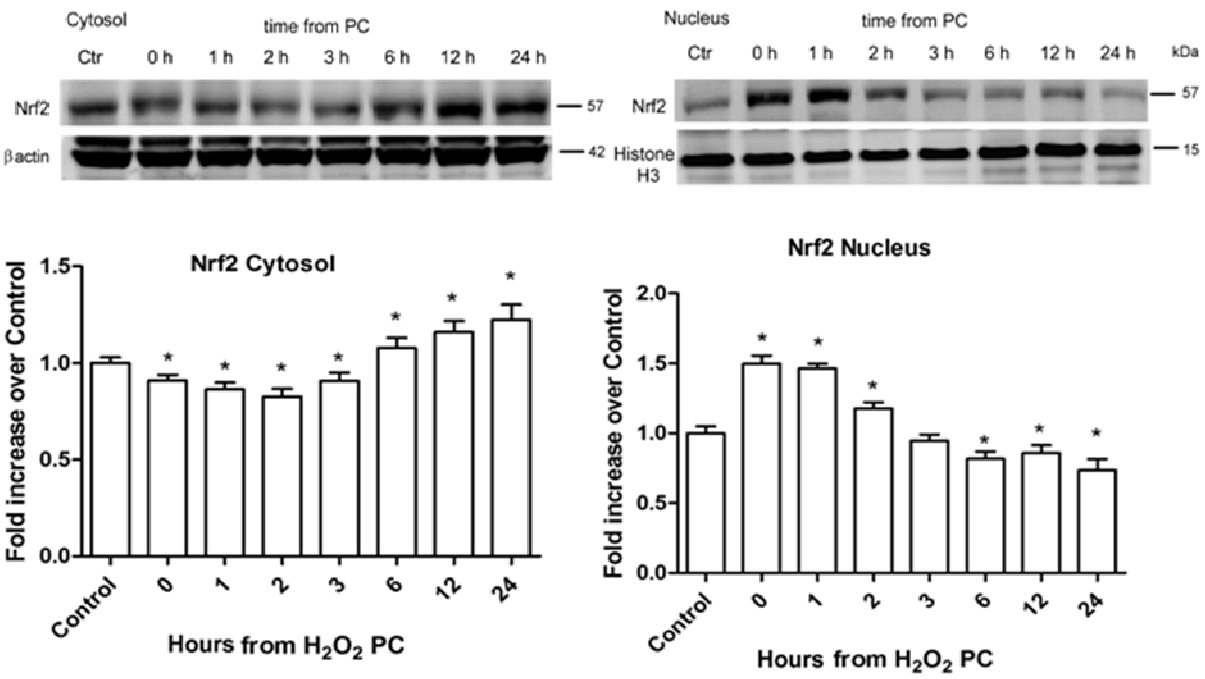


Figure 7-6: Preconditioning effect on the Nrf2 activation. *Proteins were extracted at the indicated time points following H_2O_2 PC treatment. Crude homogenates (20μg) were immunoblotted with antibodies that detect endogenous Nrf2 levels in the cytosolic and nuclear fractions. Results of scanning densotometry analysis permormed on 3 independent autoradiograph are presented. Relative amounts (means ± SD) were normalized to the intensity of β-actin (cytosolic fraction) or histone H3 (nuclear fraction) and represented as fold increase in respect to control. Data were analyzed by one-way ANOVA followed by Dunnet's test: *p< 0.05 vs Control cells*

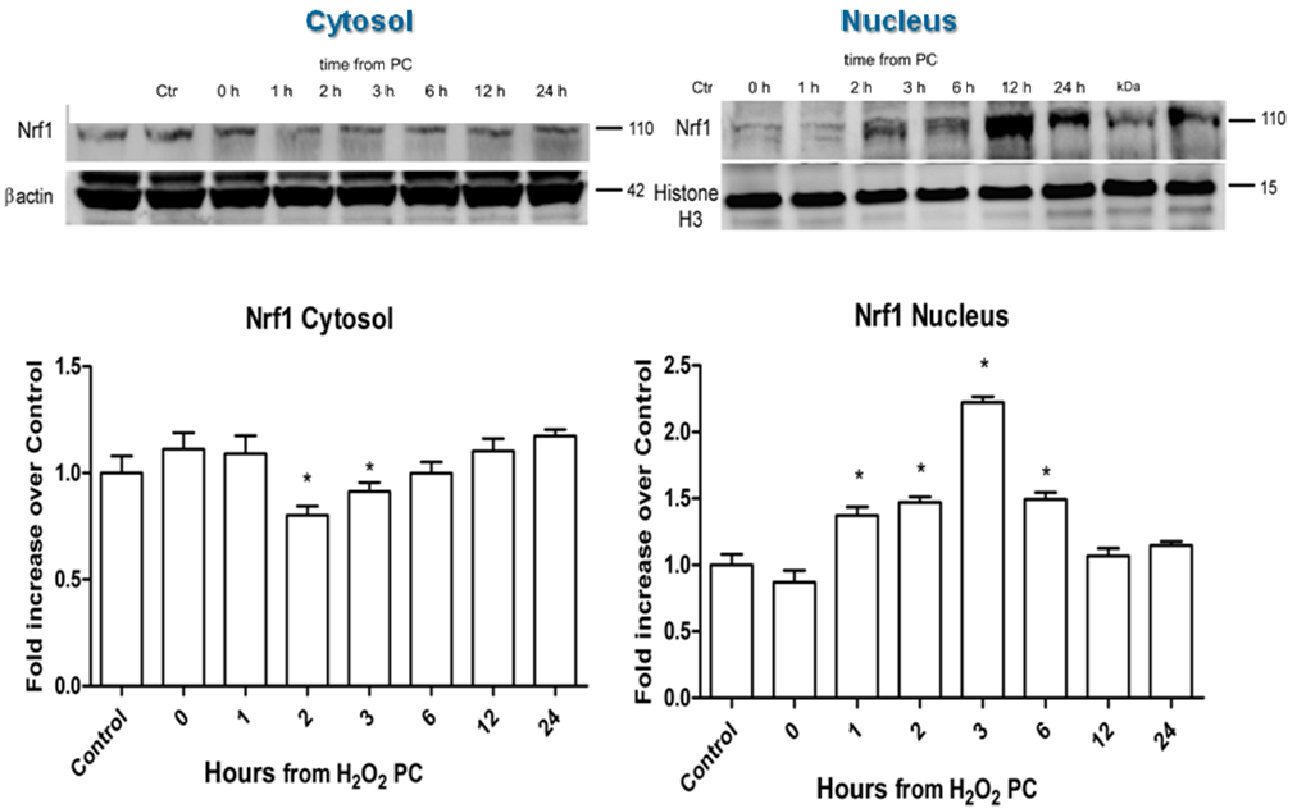


Figure 7-7: Preconditioning effect on the Nrf1 activation. *Proteins were extracted at the indicated time points following H_2O_2 PC treatment. Crude homogenates (20µg) were immunoblotted with antibodies that detect endogenous Nrf1 levels in the cytosolic and nuclear fractions. Results of scanning densotometry analysis permormed on 3 independent autoradiograph are presented. Relative amounts (means ± SD) were normalized to the intensity of β-actin (cytosolic fraction) or histone H3 (nuclear fraction) and represented as fold increase in respect to control. Data were analyzed by one-way ANOVA followed by Dunnet's test: *p< 0.05 vs Control cells*

7.1.7 Effect of Nrf1 and Nrf2 silencing on H_2O_2 PC induction of phase II enzymes.

To clarify the role of Nrf1 and Nrf2 in the induction of phase II enzymes in preconditioned cardiomyocytes, cells were transfected with Nrf1-siRNA and Nrf2 -siRNA, and scrambled control siRNA, and protein expression levels were determined 48 h posttransfection by Western blot analysis. Both Nrf1-siRNA and Nrf2-siRNA were able to strongly downregulate Nrf1 and Nrf2 (Figure 7-8 A)

In cells transfected with Nrf1-siRNA and Nrf2 -siRNA was evaluated the effect of PC on the induction of phase II enzymes (Figure 7-8 B). Cells were transfected and after 48 h were preconditioned with H_2O_2 100 μM. Proteins were extracted 24 hours after PC induction. It's interesting to note that the down-regulation of both Nrf1 and Nrf2 determines a marked reduction NQO1, CAT, GR and TRred protein level compared to non- silenced and preconditioned cells.

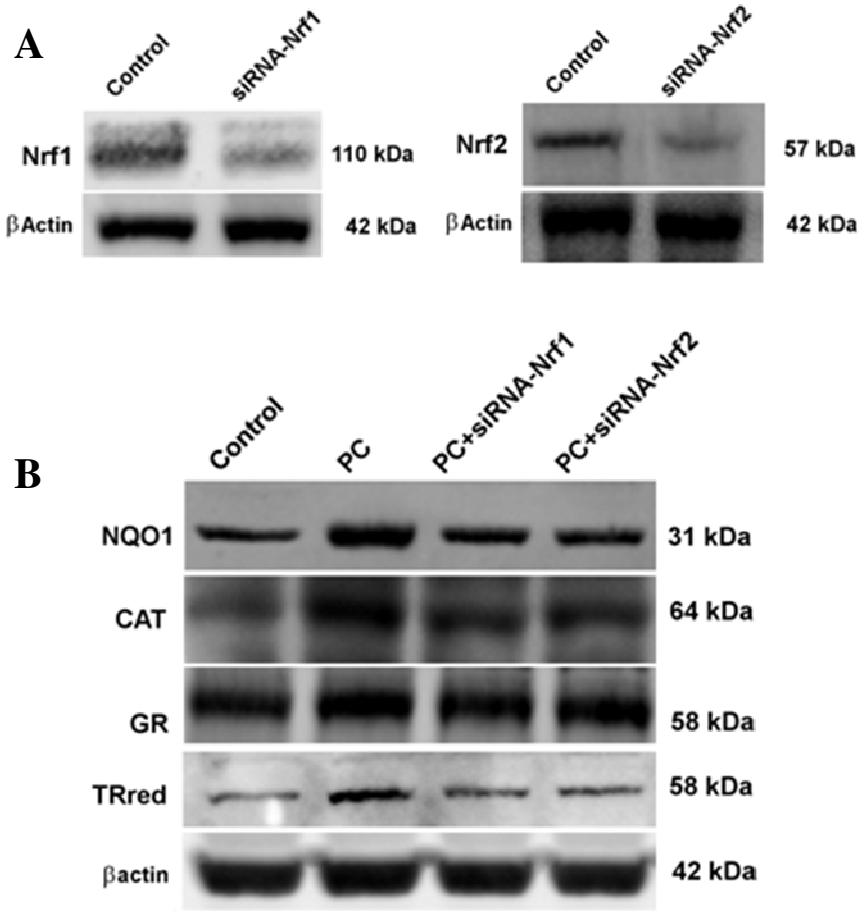


Figure 7-8: ***Role of Nrf1 and Nrf2 in H_2O_2 PC-induction of phase II enzyme.***

A)effect of Nrf1-small interfering (si)RNA and Nrf2-siRNA on Nrf1 and Nrf2 expression. Cardiomyocytes were transfected with 50 nM Nrf1-siRNA and Nrf2-siRNA and after 48 h cell lysates were immunoblotted with antibodies for Nrf1 and Nrf2 and β-actin. Representative immunoblots of 3 differentexperiments are reported. B) effect of Nrf1-siRNA and Nrf2-siRNA on NQO1, CAT, GR and TRred expression. Cardiomyocytes were transfected with 50 mM Nrf1-siRNA and Nrf2-siRNA 48 h before H_2O_2 PC. Cell lysates were immunoblotted with antibodies for NQO1, CAT, GR, TRred and β-actin. Representative immunoblots of 3 different experiments are reported.

7.1.8 Role of ERK1/2, p38 MAPK, and PI3K/Akt signaling in H_2O_2 PC.

In accordance with the widely accepted role of protein kinases in cardioprotection against many stimuli like oxidative stress, we evaluated, by immunoblot analysis, the phosphorylation, i.e. the activation, of three fundamental protein kinases: ERK1/2, p38 MAPK, and PI3K/Akt. Figure 7-9 A reports representative immunoblots of cardiomyocytes preconditioned with 1-100 μM H_2O_2 for 10 min. Phospho-ERK1/2 MAPK expression was significantly increased at any H_2O_2 concentrations, while p38 MAPK and Akt were significantly activated only by 50 and 100 μM H_2O_2. To investigate the role of these protein kinases in mediating the cardioprotection afforded by PC, we pre-treated cardiomyocytes with specific inhibitors of Akt-phosphorylation (20 μM LY), ERK1/2-phosphorylation (20 μM PD) and p38-phosphorylation (20 μM SB) for 1 h, before PC. Cell viability in the absence or presence of the different inhibitors is reported in Fig. 7-9 B. The three inhibitors alone did not influence cell viability. LY and SB significantly reversed the cardioprotective effects of PC, while PD did not modify PC protective effect against oxidative stress, suggesting a role of p38 MAPK and Akt in the cardioprotection elicited by H_2O_2-PC. To investigate the role of p38 MAPK and Akt in the induction of antioxidant and phase II enzymes, cardiomyocytes were pre-treated with LY or SB for 1 h before H_2O_2 PC and GR, TRred, CAT, and NQO1 protein level was evaluated by immunoblot analysis (Figure 7-10). LY and SB significantly inhibited the induction of GR, CAT and TRred due to

PC. Surprisingly, p38 and Akt inhibitors were not able to reduce the induction of NQO1, indicating a role of the two kinases only in the induction of GR, CAT and TRred. Enzyme activities, measured in the same experimental conditions, are in agreement with the immunoblotting data (Table 7-2). DMSO, the vehicle of PD, LY and SB, did not influence any parameters (data not shown).

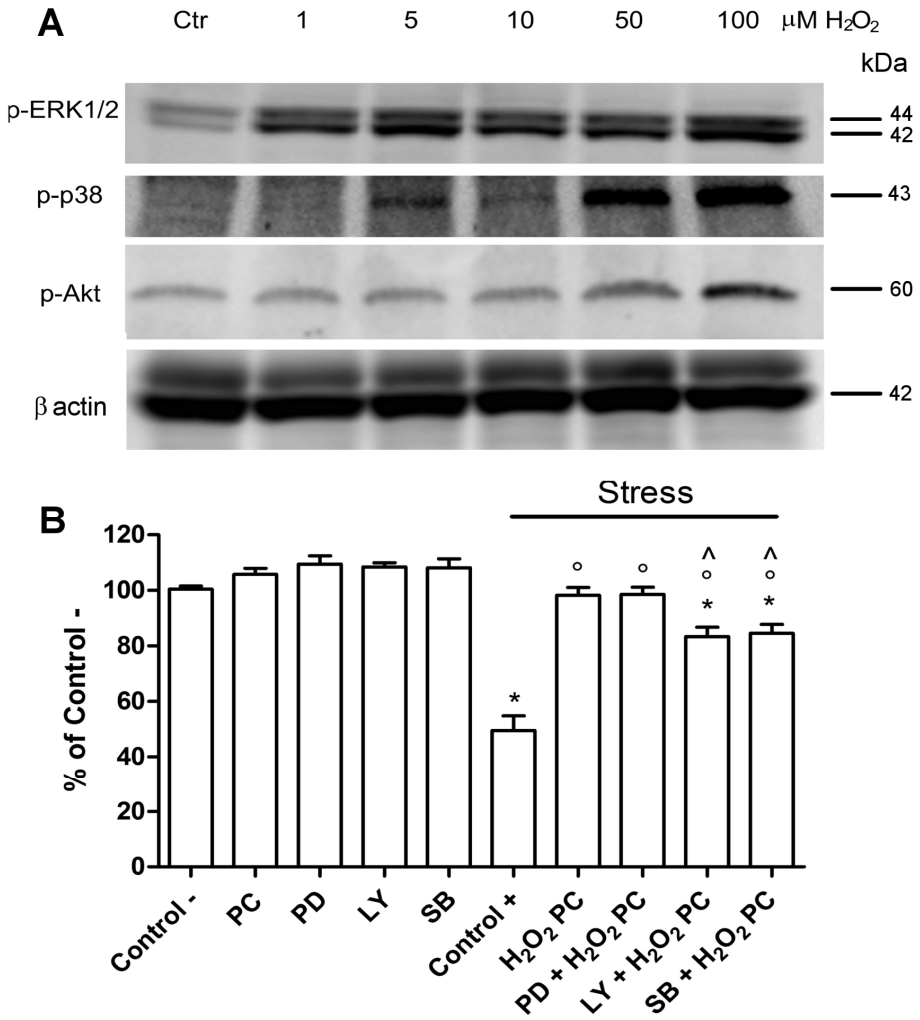


Figure 7-9: ***Role of ERK1/2, Akt, and p38 MAPK in H_2O_2 cardioprotection.***

*A: cardiomyocytes were preconditioned with 1–100 μM H_2O_2 for 10 min. Cell lysates were immunoblotted with antibodies specific for phospho-ERK1/2, phospho-p38, and phospho-Akt. Representative immunoblots of 3 different experiments are reported. B: cardiomyocytes were incubated with PD-98059 (PD), LY-294002 (LY), and SB-203580 (SB) before H_2O_2-PC and after 24h were exposed to oxidative stress. Cellular damage was assessed by MTT assay and reported as %cell viability compared with control_. Each bar represents the means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *P < 0.05, with respect to control -;*

°P < 0.05, with respect to stress (control +); ^P < 0.05, with respect to H_2O_2-PC.

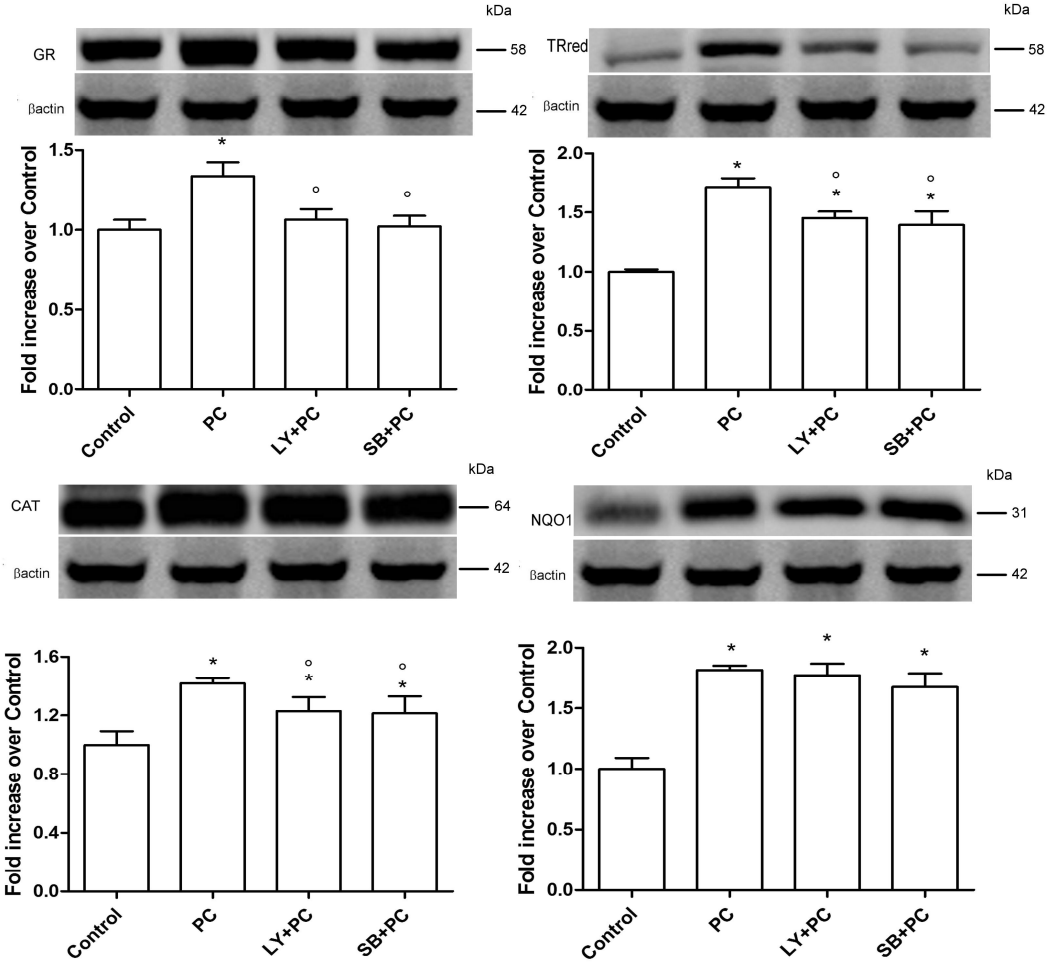


Figure 7-10: ***Role of Akt and p38 MAPK in H_2O_2-PC induction of phase II enzymes.*** *Cardiomyocytes were incubated with LY or SB before H_2O_2-PC. Cell lysates were immunoblotted with antibodies for GR, TRred, CAT, and NQO1. Representative immunoblots of 3 different experiments are reported. White line inserted in NQO1 blot represents a skipped line. Results of scanning densitometry analysis performed on 3 independent autoradiographs are presented. Relative amounts (means ± SD) were normalized to the intensity of the same β-actin blot and represented as fold increase in respect to controls. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *P < 0.05, with respect to control; °P < 0.05, with respect to PC.*

Table 7-2: ***Effects of LY and SB on GR, TRred, CAT, and NQO1 activities after H_2O_2-PC***

	GR (mU/mg protein)	TR (nmol/min/mg protein)	CAT (nmol/min/mg protein)	NQO1 (mU/mg protein)
Control	10.92±1.18	52.72±1.22	39.12±2.73	10.17±1.54
PC	14.48±0.36*	76.77±5.94*	63.87±5.54*	20.38±2.85*
LY	10.421±0.62°	54.54±2.01°	40.10±1.45°	10.00±1.10°
SB	10.14±0.79°	49.19±6.96°	38.41±3.39°	19.56±0.26°
LY+PC	10.87±1.32°	64.25±3.59*°	49.62±1.34*°	20.54±2.75*
SB+PC	11.78±1.04°	65.13±7.53*°	49.95±4.95*°	19.87±1.42*

*Value represent means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. Cardiomyocytes were incubated with LY-294002 (LY) or SB-203580 (SB) before H_2O_2 preconditioning (PC), and after 24 h cells were lysed for enzymatic activity measures. *$P < 0.05$, with respect to control; °$P < 0.05$, with respect to PC.*

7.2 Effects of SF on carbonyl stress induced by MG in cardiomyocytes

7.2.1 Effects of methylglyoxal on cell viability in cardiomyocytes

The first point was to evaluate the potential cytotoxicity of methylglyoxal (MG) and to determine the IC_{50} value in primary cultures of neonatal rat cardiomyocytes.

Cells were treated with increasing MG concentrations (0.1-5 mM) and the MTT cell viability assay was assessed after 24 h (fig 7-11).

At the lowest MG concentrations (0.1-0.5 mM) cell viability was comparable to those of control cells, while 0.75-5 mM MG siginficantly reduced cell viability compared to control cells. In particular, at 1.0 mM cell viability was 50% lower than control cells. We decided to choose the concentration of 1.0 mM as an IC_{50} value in subsequent experiments.

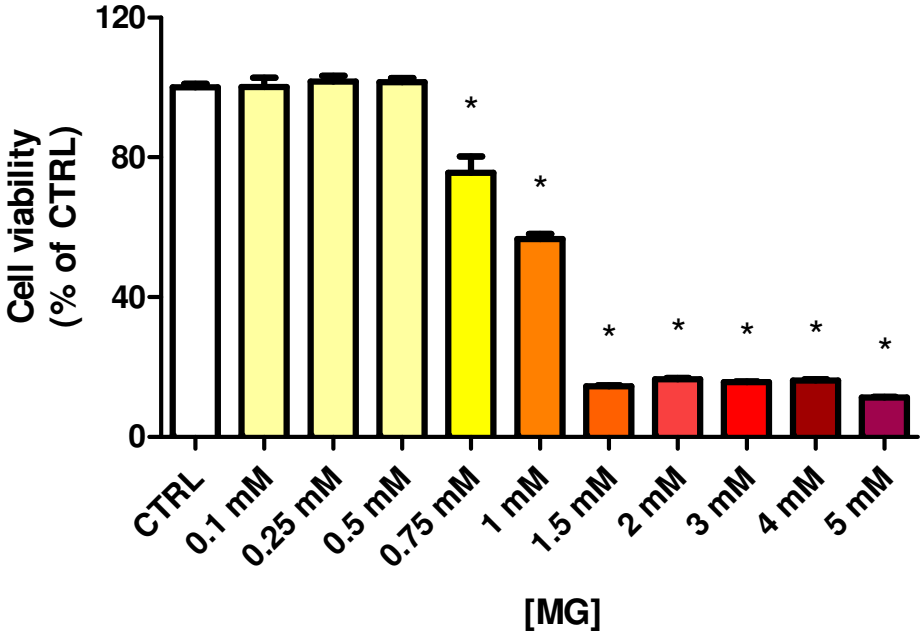


Figure 7-11: ***Effect of MG on cell viability of cultured cardiomyocytes.*** *Cells were treated with MG (0.1-5 mM) for 24 h. Cell viability was assessed by the MTT assay and reported as % cell viability compared with Control. Each bar represents means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's ttest: *p< 0.05 vs Control*

7.2.2 SF protection against MG-induced damage

Subsequently, we evaluated the possible protective effect of SF against carbonyl stress induced by MG. Cardiomyocytes were pre-treated with 5µM SF for 24 h, exposed to MG 1 mM and after 24 h cell viability was determined by MTT assay (figure 7-12). 5 µM SF was chosen because it has been previously shown that this is the minimum concentration able to evoke the maximum protective effect

against oxidative stress in (Angeloni et al. 2009). Treatment with SF significantly increased cell viability compared to cells exposed only to MG (Fig. 7-12); although cell viability was significantly lower compared to that measured in control cells.

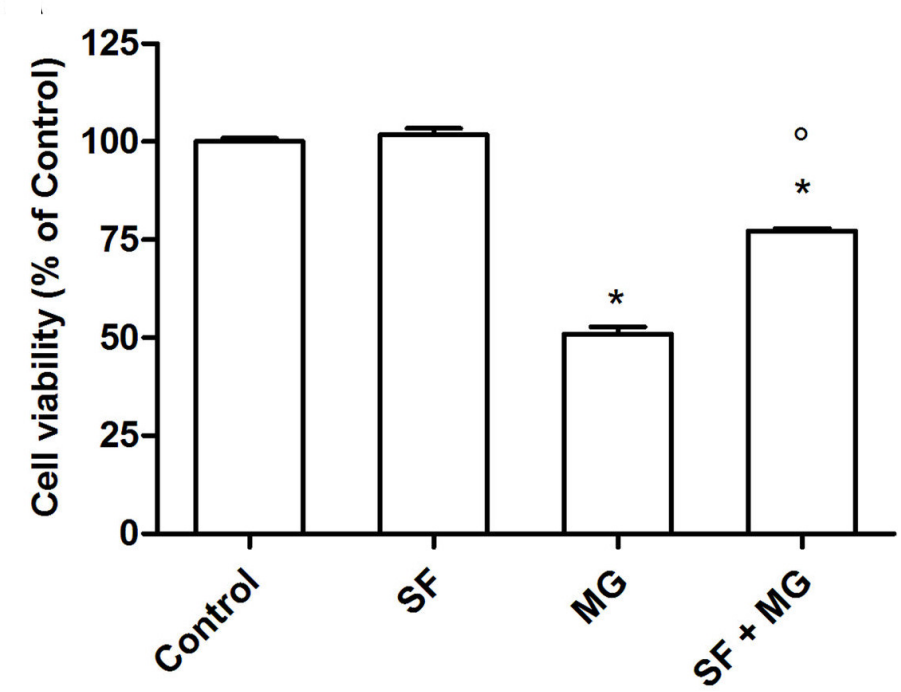
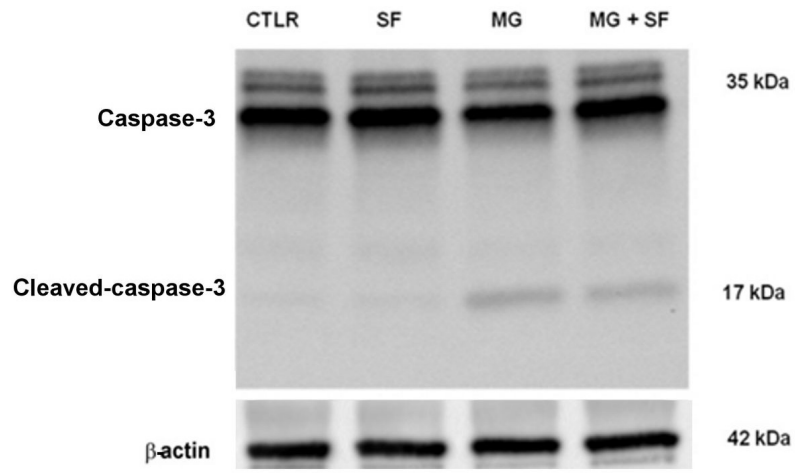


Figure 7-12: ***Protective effect of SF against carbonyl stress induced by MG.*** *Cells were pre-treated with 5µM SF for 24 h before the addition of 1mM MG. Cell viability was assessed by the MTT assay and reported as % cell viability in comparison to control. Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

To better understand the fate of cells exposed to MG, we evaluated the activation and activity of caspase-3. Cells were pre-treated with 5 μM SF for 24 h, exposed to 1 mM MG and after 24 h cells were lysed and proteins extracted for immunoblotting or caspase 3 activity (Figure 7-13). Results indicate that MG increase protein content of caspase 3 active form (17 kDa fragment) and concurrently decrease caspase 3 pro-inactive form in respect to Control. In agreement with MTT data, pre-treatment with SF reduces the formation of the low molecular weight fragment in MG exposed cells, indicating that this compound is able to counteract apoptotic cell death induced by MG. To confirm these results, caspase 3 activity has been evaluated in the same experimental conditions (Fig. 7-13 B). Exposure to MG causes a significant increase in the activity of the enzyme compared to control cells, meanwhile pre-treatment with 5 μM SF significant decrease caspase 3 activity in MG treated cells compared to cells exposed to MG but not pre-treated with SF.

a)



b)

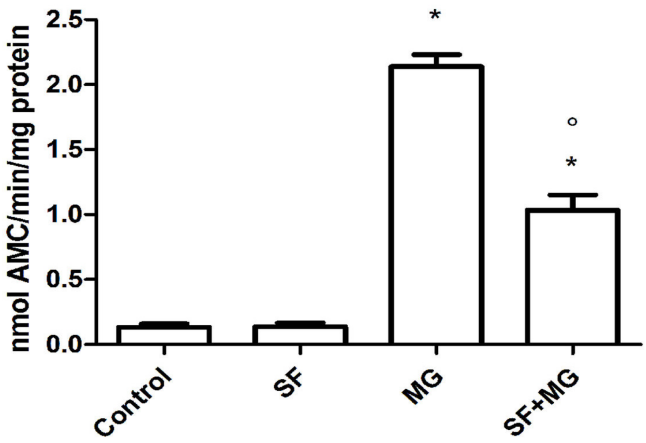


Figure 7-13: ***SF protective effects against MG-induced apoptosis in cardiomyocytes*** *Cells were pre-treated with 5μM SF for 24 h before the addition of 1mM MG. a) Cell lysates were immunoblotted with caspase-3 antibody that detects both full length caspase-3 (35kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa). b) Caspace-3 activity was measured spectrofluorimetrically by hydrolysis of the peptidesubstrate Ac-DEVD-AMC. Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.2.3 SF effect against MG-induced ROS production in cardiomyocytes

GLO1 is a GSH-dependent enzyme and excess MG presents serious toxic effects since it depletes GSH via a covalent bond. As GSH is one of the most important endogenous antioxidant molecules, its depletion could be related to an increase in ROS. To verify if SF is able to counteract oxidative stress in MG exposed cardiomyocytes we used 3 different probes, DCHF-DA, DHE, and MitoSOX to measure intracellular ROS production. Representative confocal fluorescent micrographs of cardiomyocytes pre-treated with 5 μM SF for 24 h, exposed to 1 mM MG and stained with DHE or MitoSox are shown in figure 7-14. MG induced a marked increase in DHE and MitoSox fluorescence intensity, while SF was able to maintain fluorescence intensity to value comparable to control cells. SF alone had no effect on fluorescence intensity of cells stained with DHE and MitoSox.

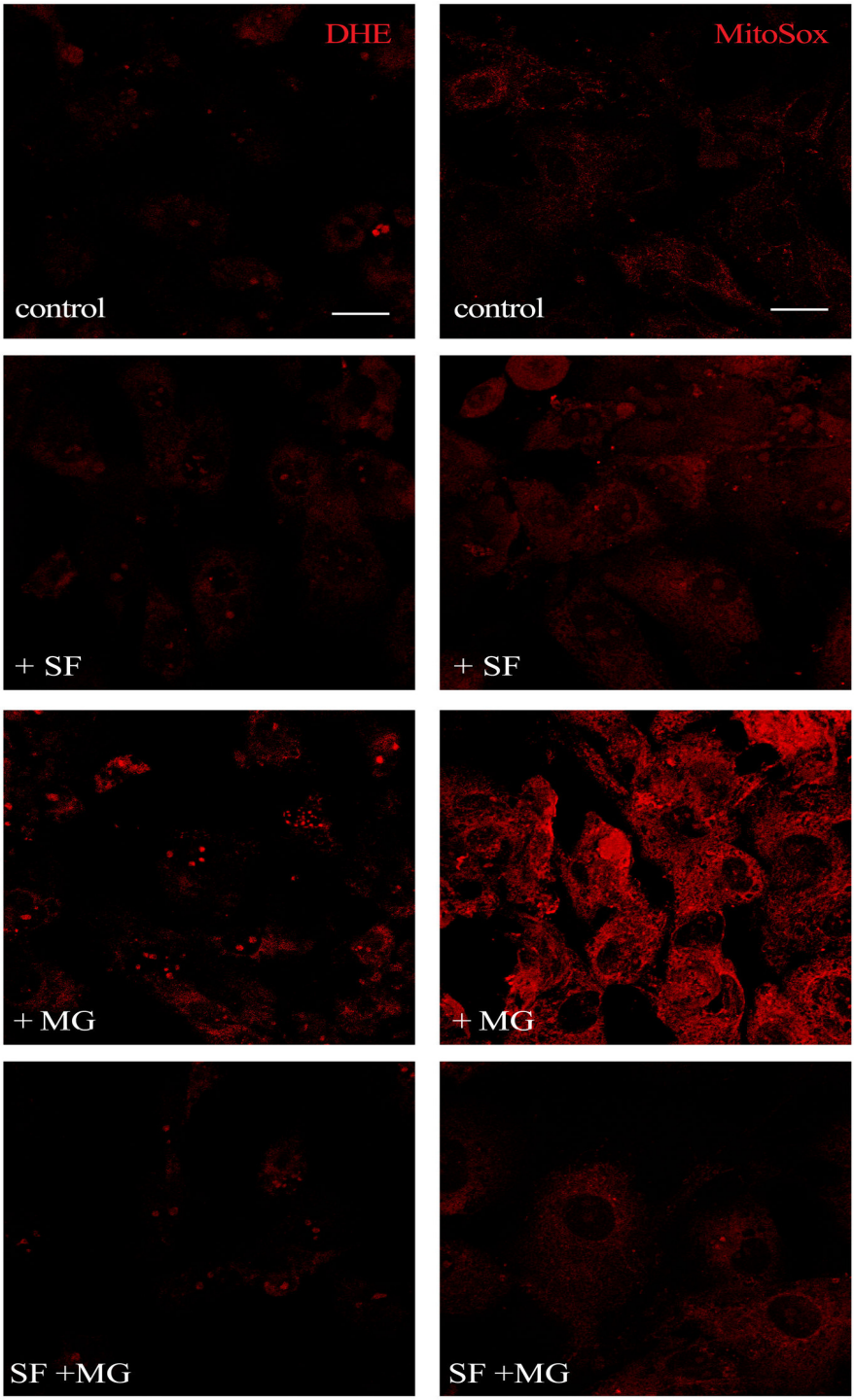


Figure 7-14: ***Confocal microscopy analysis of SF effect on MG-induced ROS production.*** *Cardiomyocytes were treated with 5 µM SF for 24 h before the addiction of 1mM MG. Cytosolic ROS production was measured using DHE and mitochondrial ROS production by MitoSOx staining. Scale bar 10 µm*

Semi-quantitative measurements by fluorescent plate reader of DCHF-DA, DHE and MitoSox stained cardiomyocytes are reported in figure 7-15. MG exposure induced a significant increase in intracellular ROS levels. In particular, the highest increase was obtained with DCHF-DA, the less specific probe able to detect non only H_2O_2 but also several one-electron-oxidazing species (Kalyanaraman et al. 2011)(Fig.7-15a). In agreement with the results obtained by confocal microscopy, MG treatment significantly increased the fluorescence intensity of DHE and MitoSox that more specifically target superoxide (Fig.7-15b and c). SF was able to counteract the intracellular ROS level increase induced by MG, maintaining fluorescence intensity of the 3 probes to level comparable to control cells.

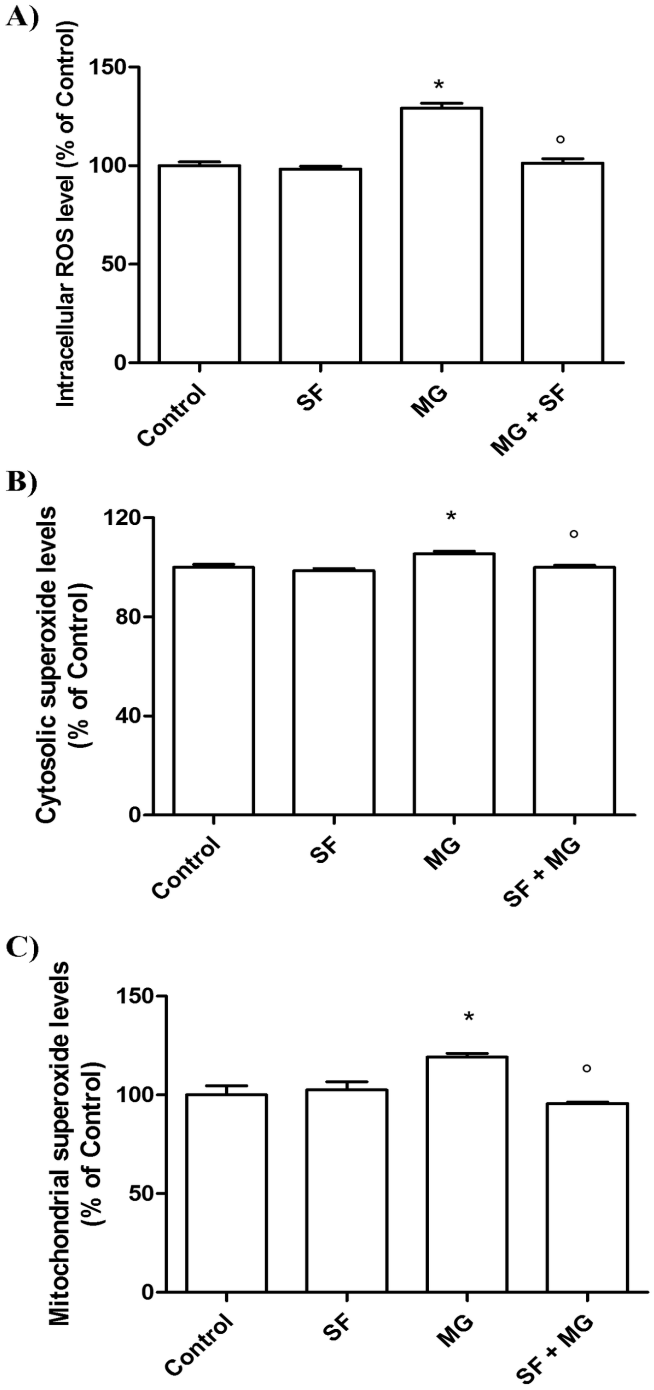


Figure 7-15: ***Fluorimetric assays of SF effect on MG-induced ROS generation.*** *Cardiomyocytes were treated with 5µM SF for 24 h before addition of 1 mM MG. A)Intracellular ROS levels were determined with the peroxide-sensitive probe DCHF-DA. B)Cytosolic ROS levels were determined with DHE. C)Mitochondrial ROS levels were determined with MitoSox. Each bar represent means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

To better elucidate SF role in counteracting oxidative stress, total antioxidant activity (TAA) was evaluated by ABTS assay (Fig.7-16). SF alone was able to significantly increase TAA in respect to control cells, while MG significantly reduced this value. Interestingly, SF was able to restore TAA levels of MG treated cardiomyocytes to values comparable to control cells.

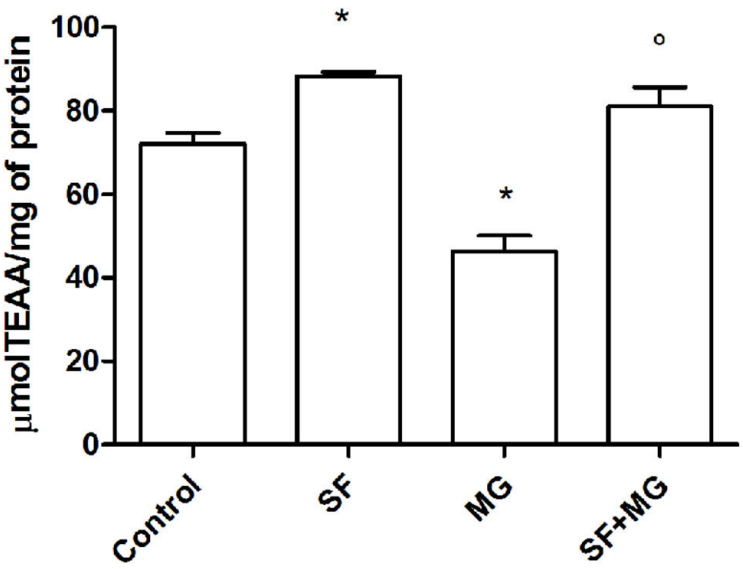


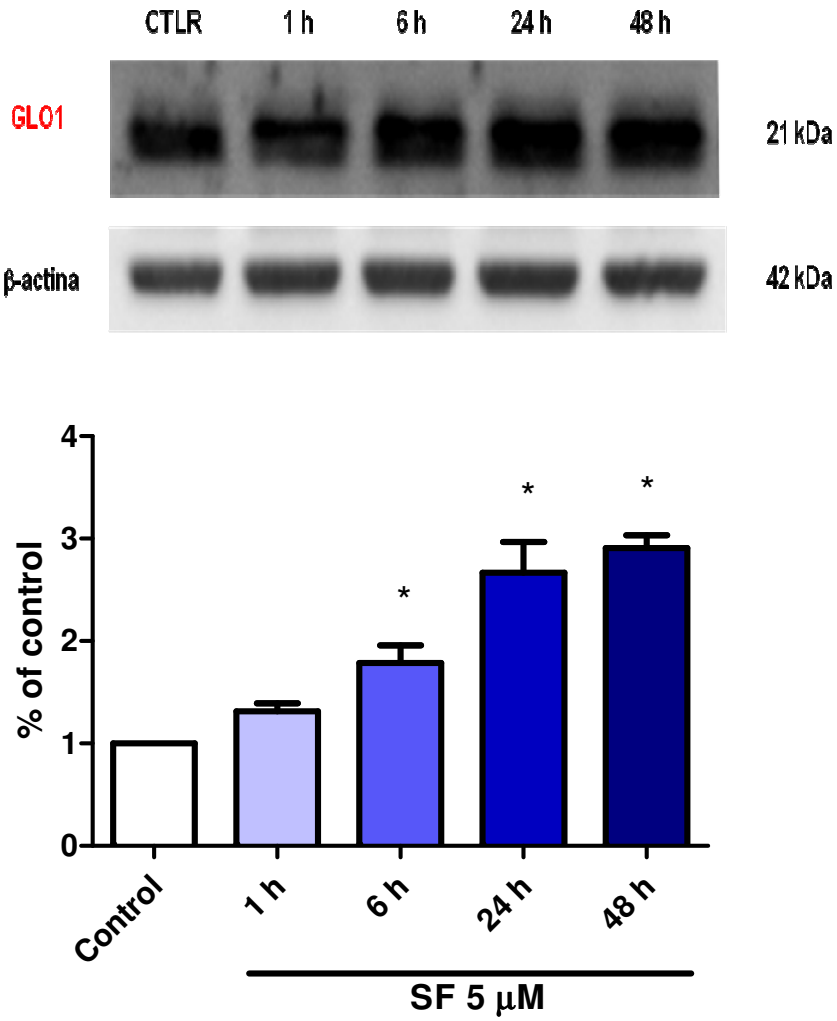
Figure 7-16: ***SF effects on cardiomyocytes total antioxidant activity.*** *Cardiomyocytes were treated with 5 µM SF for 24 h before addition of 1 mM MG. Cell lysates were submitted to the ABTS radical cation decolorization assay and the antioxidant activity of the cells were expressed as mean ± SD of µmol of trolox antioxidant activity per mg of protein. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.2.4 Modulation of GLO1 by SF

Recently it has been shown that the upregulation of GLO1 enzyme is modulated by Nrf2, in a manner similar to what happens for the antioxidant / detoxifying phase II enzymes. Since SF is able to induce the translocation of Nrf2 from the cytosol to the nucleus, thereby regulating phase II enzymes, we hypothesized that it might be also able to up-regulate GLO1 . To test this hypothesis, we evaluated the effect of SF on GLO1 expression and activity.

Cells were treated with 5 μM SF for different time points (Figure 7-17). GLO1 protein expression significantly increases compared to that measured in control cells after 6 h SF treatment, and this induction becomes even more pronounced after 24-48 hours of treatment (Figure 7-17a). To relate GLO1 overexpression to its functional activity, cardiomyocytes were treated with 5 μM SF and GLO1 enzymatic activity was measured at different time points (1-48h) (Figure 17-7 b). After 1 and 6 h GLO1 activity was slightly increased but not significantly different from control cells. After 24 and 48 h SF was able to significantly increase GLO1 activity with respect to untreated cells.

a)


CTLR
1 h
6 h
24 h
48 h
GLO1
21 kDa
β-actina
42 kDa
% of control
4
3
2
1
0
*
*
*
Control
1 h
6 h
24 h
48 h
SF 5 µM

b)

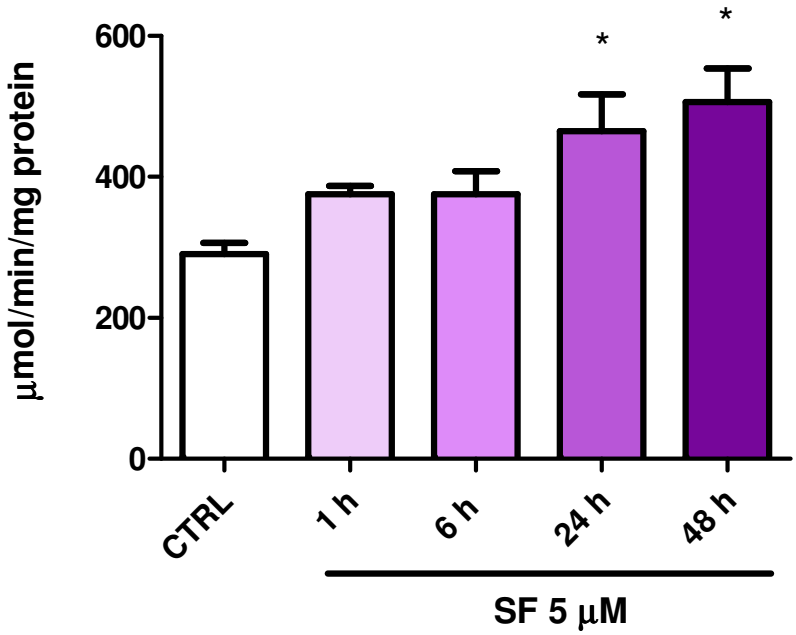


Figure 7-17: ***Effects of SF on protein expression and enzymatic activity of GLO1 in cardiomyocytes exposed to MG.*** *Cells were treated with 5 µM SF for different time (1, 6, 24, 48 h). a)protein expression was assessed by immunoblotting analyses. Results of scanning densitometry analysis performed on 3 independent autoradiographs are presented. Relative amounts (means ± SD) were normalized to the intensity of the same β-actin blot and represented as fold increase in respect to controls. b) enzymatic activity of GLO1 was assessed by spectrofotometric assay. Each bar represent means ± SD of three independent experiments. Data was analyzed by one-way ANOVA followed by Dunnet's test: *p< 0.05 vs Control cells*

7.2.5 Effects of SF on AGEs formation in cardiomyocytes exposed to MG

Finally, we evaluated the effect of SF on the production of AGEs. Cells were pre-treated with 5 μM SF for 24 hours and subsequently exposed to 1 mM MG for 24 hours and AGE levels were measured with an ELISA assay able to identify the main AGEs. In cells not treated with SF the exposure to MG caused a significant increase in the production of AGEs compared to control cells (Fig.7-18). Pre-treatment with SF, in contrast, significantly reduced the formation of AGEs compared to cells exposed to MG, with values very similar to control cells

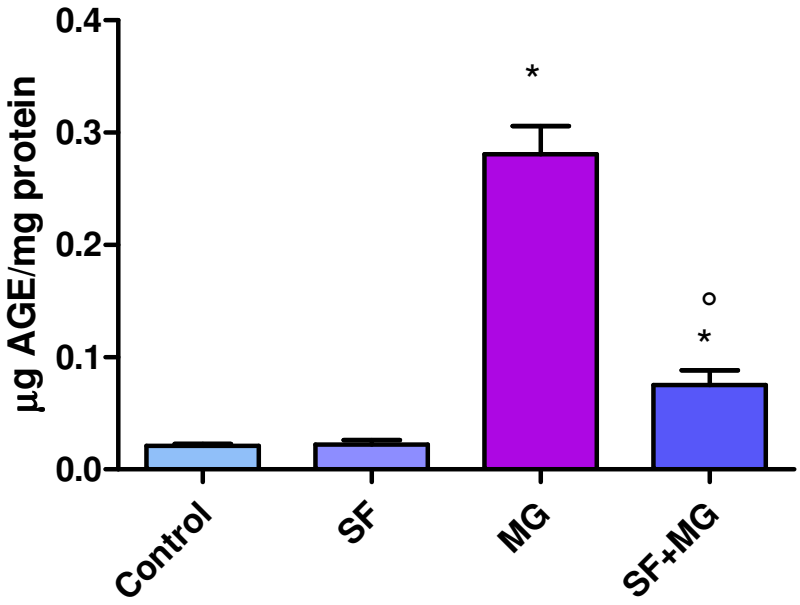


Figure 7-18: ***Effects of SF on AGEs formation in cardiomyocytes exposed to MG.*** *Cardiomyocytes were treated with 5 µM SF for 24 h before addition of 1 mM MG, and AGE formation was evaluated by AGE-ELISA using a specific anti-AGE antibody. Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.3 Effects of SF on carbonyl stress induced by MG in SH-SY5Y cell line

7.3.1 Effects of methylglyoxal on cell viability in SHSY-5Y cell line

To evaluate the potential cytotoxicit effect of MG, SH-SY5Y cells were treated with increasing concentrations (0.1-5 mM) of MG for 24 h, after which cell viability was assessed by MTT assay (Figure 7-19). At the concentrations 0.25-5 mM, MG induced a significant reduction of cell viability compared to control cells. In particular, 0.5 mM MG reduced cell viability by 50% compared to the control (IC_{50}).

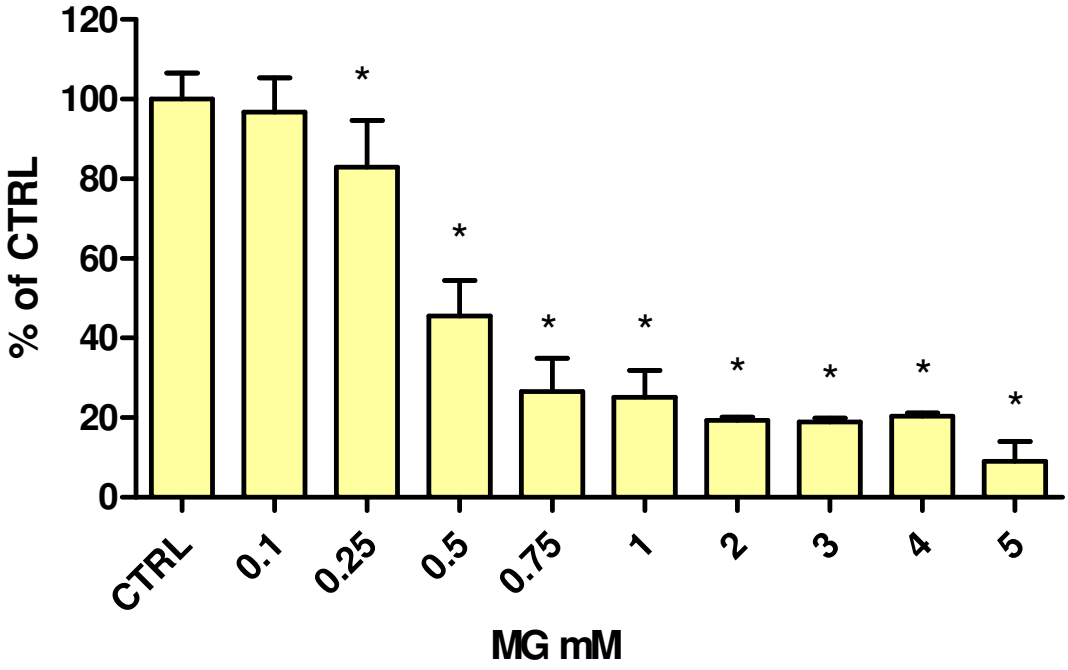


Figure 7-19: ***Effect of MG on cell viability in SHSY-5Y cell line.*** *Cells were treated with MG (0.1-5 mM) for 24 h. Cell viability was assessed by the MTT assay and reported as % cell viability compared with controls. Each bar represents means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test: *p< 0.05 vs Control cells*

7.3.2 SF protective effects against MG-induced damage in SHSY-5Y cell line

The next step was to evaluate the possible protective effect of SF against glycative damage induced by MG in SH-SY5Y neuroblastoma cell line.

Cells were treated with 5 µM SF for 24 h and then exposed to 0.5 mM MG for 24 h. Cell viability was assessed by MTT assay (Figure 7-20). As previously reported, MG induced a significant reduction of cell viability by about 50% compared to control cells. SF treatment significantly increased cell viability compared to cells exposed to MG, even if cell viability was significantly lower compared to that measured in control cells. To confirm these data, LDH release was measured evaluating LDH activity in the culture medium (Figure 7-21). SH-SY5Y were treated with 5 µM SF for 24 h and then exposed to 0.5 mM MG for 24 h. As expected, MG treatment induced a significant and strong increase of LDH release, while SF treatment was able to maintain LDH activity at level comparable to control cells. Moreover, to better understand the fate of cells exposed to MG, we evaluated the activity of the pro-apoptotic enzyme caspase-3in the same experimental conditions (figure 7-22). MG significantly increased caspase-3 activity in SH-SY5Y, indicating that at least part of the cells undergo apoptotic cell death, while SF treatment significantly reduced caspase-3 activity compared to cells exposed to MG. Interestingly, in MG treated cells SF was able to maintain both LDH release and caspase 3 activity to value comparable to control cells.

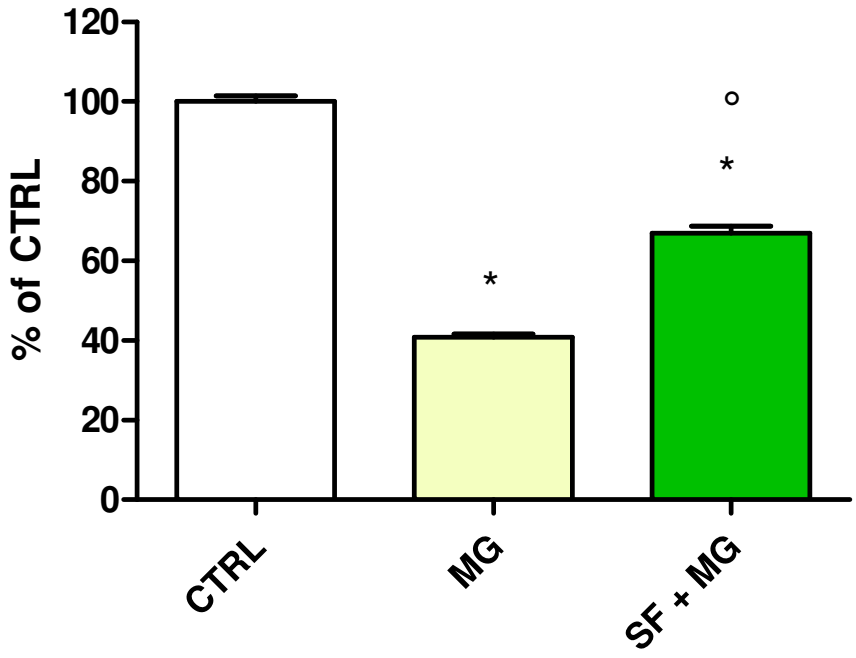


Figure 7-20: ***Protective effect of SF against carbonyl stress induced by MG in SHSY-5Y cell line.*** *Cells were treated with 5µM SF for 24 h before the addition of 0.5 mM MG. Cell viability was assessed by the MTT assay and reported as % cell viability in comparison to control. Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

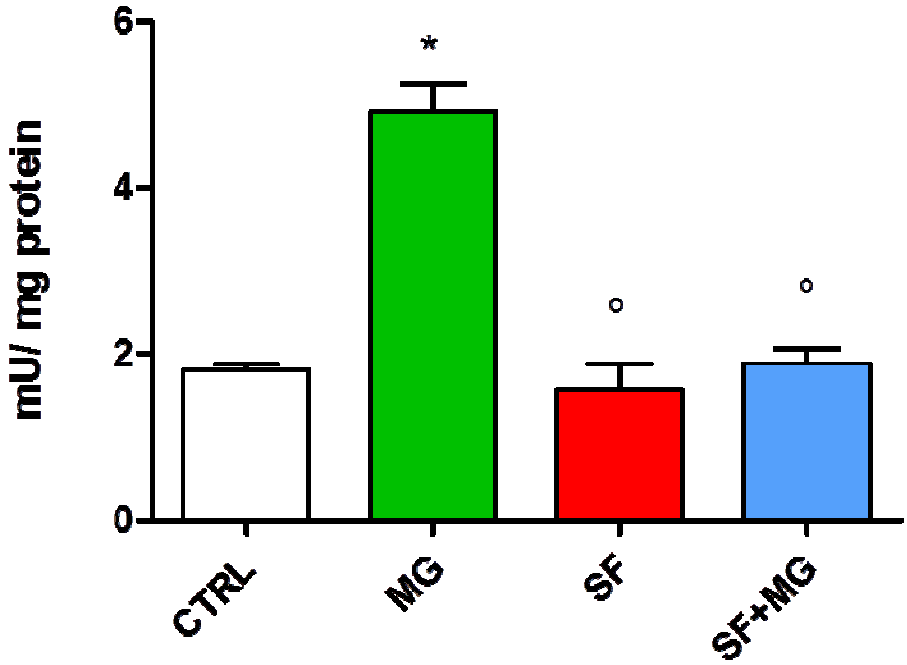


Figure 7-21: ***Effects of SF on LDH activity in cardiomyocytes exposed to MG.*** *Cells were treated with 5μM SF for 24 h before the addition of 0.5 mM MG. LDH activity was assessed by spectrometric assay and reported as mU/mg of protein Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

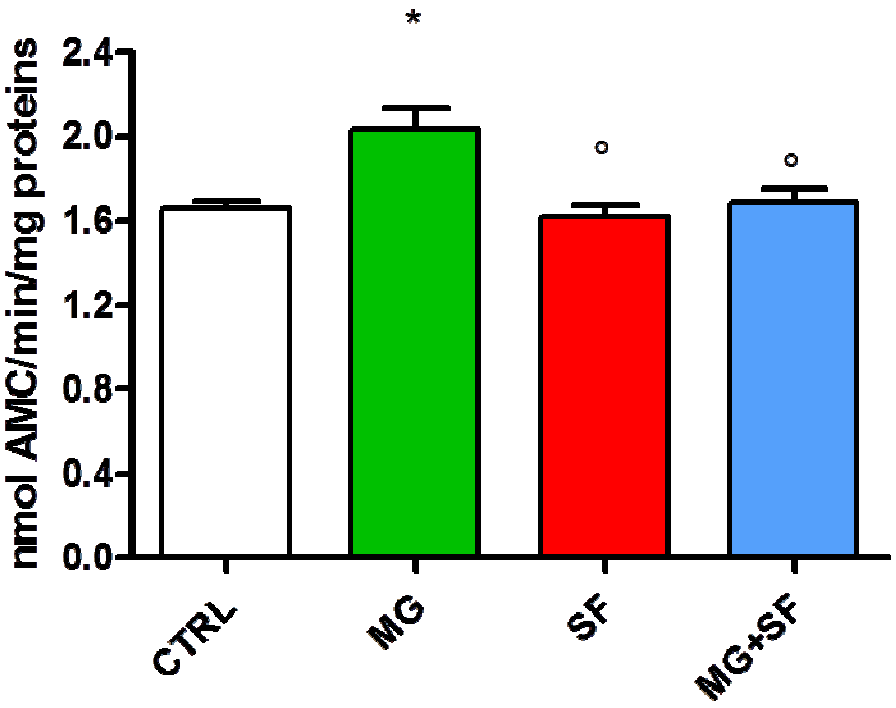


Figure 7-22: SF protective effects against MG-induced apoptosis in SH-SY5Y cell line. *Cells were treated with 5μM SF for 24 h before the addition of 0.5 mM MG. Caspace-3 activity was measured spectrofluorimetrically by hydrolysis of the peptide substrate Ac-DEVD-AMC. Each bar represent means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.3.3 SF effects against MG-induced ROS production in SH-SY5Y

Since it has been demonstrated that MG induces oxidative stress in neuronal cells (Angeloni et al. 2013) and that SF exerts its protective action through the induction of antioxidant/ detoxifying phase II enzymes, we investigated SF ability to counteract MG-induced intracellular ROS production by the DCFH-DA assay. Cells were treated with μ5 M SF for 24 hours and then exposed to 0.5 mM MG for 6 hours (Fig.7-23).

In cells not treated with SF, MG exposure resulted in a significant increase in intracellular ROS production compared to control cells. SF treatment, in contrast, significantly reduced the formation of intracellular ROS compared to cells exposed to MG.

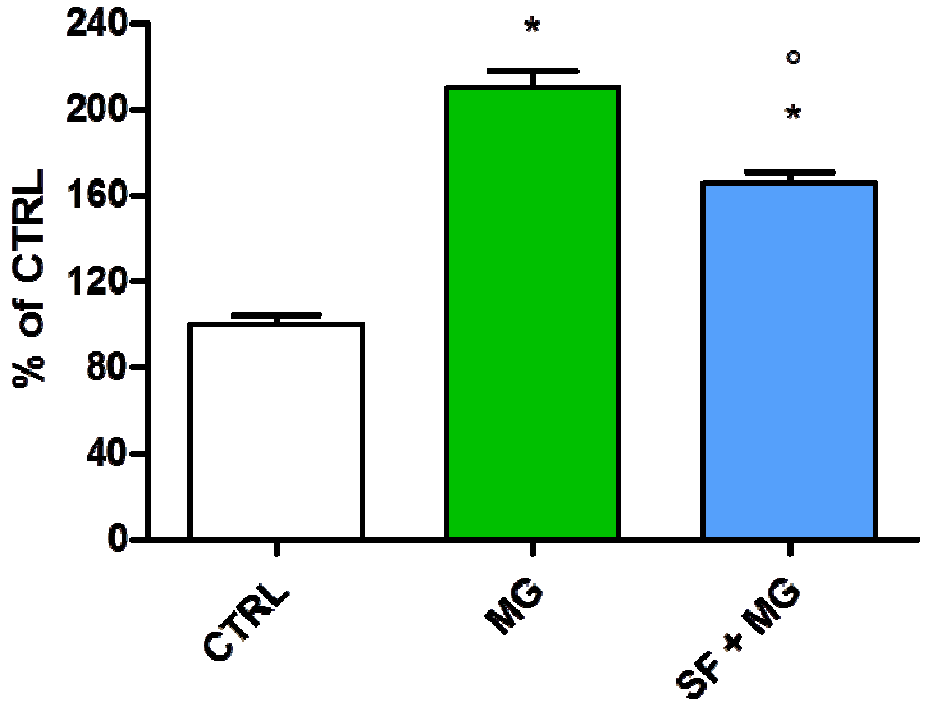


Figure 7-23: ***Effects of SF on intracellular ROS formation in SHSY-5Y cell line exposed to MG.*** *Cells were treated with 5 µM SF for 24 h before the addition of 0.5 mM MG. Intracellular ROS levels were determined with the peroxide-sensitive probe DCHF-DA. Each bar represent means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Dunnets's test: *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.3.4 *Effects of SF on intracellular levels of reduced GSH in SHSY-5Y exposed to MG*

A reduction in intracellular levels of reduced GSH occurs in conditions of oxidative stress, so we evaluated the effect of SF on intracellular reduced GSH levels by the MCB assay. Cells were treated with 5 μM SF for 24 h and exposed to 0.5 mM MG (Fig.7-24). SF alone was able to increase reduced GSH levels compared to control cells. Exposure to MG caused a significant decrease in GSH levels compared to control cells, meanwhile treatment with SF significantly increase the amount of reduced GSH compared to control cells.

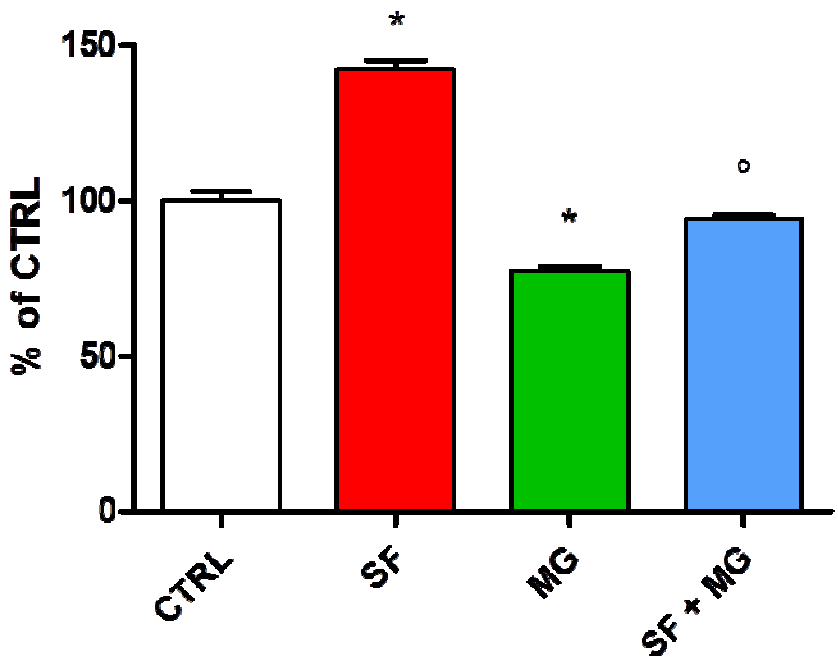


Figure 7-24: ***Effects of SF on intracellular levels of reduced GSH in SHSY-5Y cell line exposed to MG.*** *Cells were treated with 5 µM SF for 24 h before addition of 0.5 mM MG. Reduced GSH levels were assessed by spectrofotometric assay MCB and reported as % respect to controls. Each bar represents means ± SD of 4 independet experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test: : *p< 0.05 vs Control cells; °p< 0.05 vs MG*

7.3.5 Effects of SF on protein expression of GLO1 in SHSY-5Y cell line exposed to MG

The glyoxalase system is the most important pathway for the detoxification of MG. It comprises two enzymes: GLO1 and GLO2. The first catalyzes the conversion of the hemithioacetal, formed by the nonenzymatic reaction of GSH with MG, to S-D-lactoylglutathione. GLO2 converts S-D-lactoylglutathione to D-lactate, which recycles GSH in the process (Thornalley 1993). Recently it has been demonstrated that GLO1 is up-regulated through the Nrf2/ARE pathway (Xue et al. 2011). As previously underlined, numerous studies have highlighted the role of SF as a potent natural phase II inducer in different cell models. As phase II enzymes are modulated thorough Nrf2/ARE pathway, we have hypothesized that SF could modulate also GLO1. Cells were treated with 5 μM SF for different time points (1, 6, 24, 48 h) and and GLO1 activity was measured with a spectrophotometric assay (figure 7-25). After 1 and 6 h GLO1 activity was slightly increased but not significantly different from control cells. After 24 and 48 h SF was able to significantly increase GLO1 activity with respect to control cells.

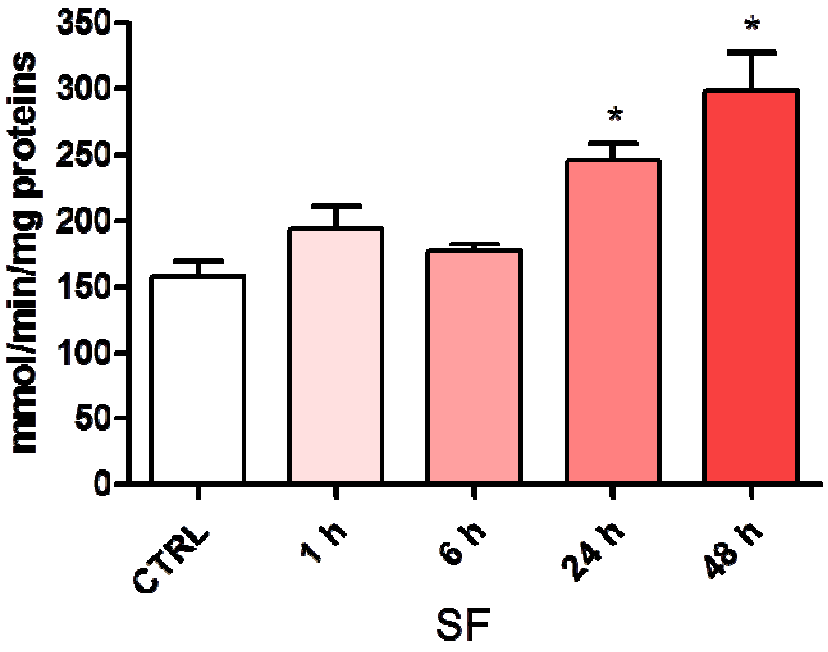


Figure 7-25: ***Effects of SF on GLO1 activity in SH-SY5Y cell line exposed to MG. .*** *Cells were treated with 5 μM SF for different periods (1, 6, 24, 48 h). Enzymatic activity of GLO1 was assessed by spectrofotometric assay. Each bar represent means ± SD of three independent experiments. Data was analyzed by one-way ANOVA followed by Dunnett's test: *p< 0.05 vs Control cells*

7.3.6 Protein kinases modulation by MG

Since we observed that MG induces apoptotic death in SH-SY5Y cells, we hypothesized that MG could modulate some of the signal transduction pathways involved in the induction of apoptotic cell death

To test our hypothesis, cells were treated with 0.5 mM MG and after time points, the activation (phosphorylation) of p38 MAPK, JNK and Akt was evaluated by immunoblotting analisys (figure 7-26).

p38 MAPK and JNK phosphorylation was significantly increased already at 30 min and lasted until 24 h. On the other hand, the phosphorylation of the anti-apoptotic kinase Akt was strongly reduced at 2, 6 and 24 h.

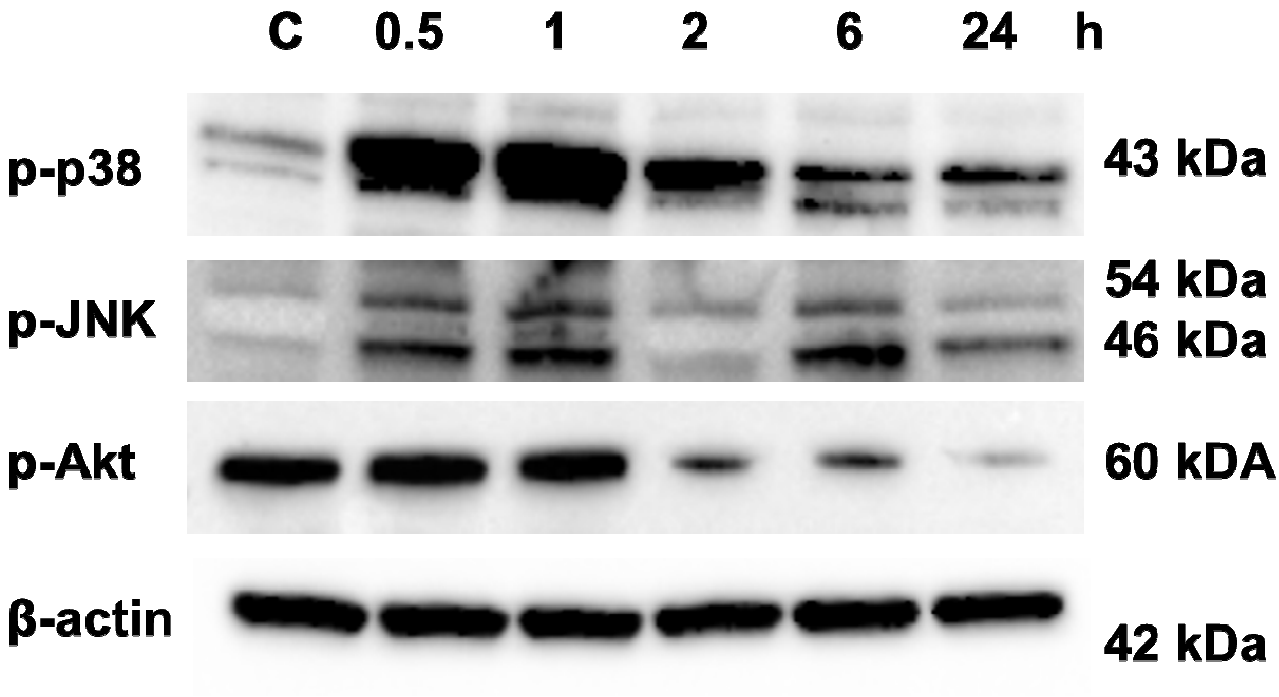


Figure 7-26: ***Effects of SF on protein expression and enzymatic activity of GLO1 in cardiomyocytes exposed to MG.*** *Cells were treated with 0.5 mM MG for different time (0.5, 1, 2, 6, 24 h). Cell lysates were immunoblotted with antibodies for p-p38, p-JNK and p-Akt.*

As MG was able to modulate these signaling pathways, we subsequently investigated the possible SF effect on the phosphorylation of these kinases. Cells were treated with SF for 24 h and then exposed to MG for 30 min, to evaluate p38 and JNK activation, to MG for 2 h to study Akt activation (figure 7-27). SF treatment had no effect on the phosphorylation of p38 and JNK in the absence of MG, whereas in cells exposed to 0.5 mM MG, SF was able to slight reduce the activation of both kinases compared to cells not treated with SF and exposed to MG.

On the other hand SF significantly increased Akt activation both in the presence and in absence of MG.

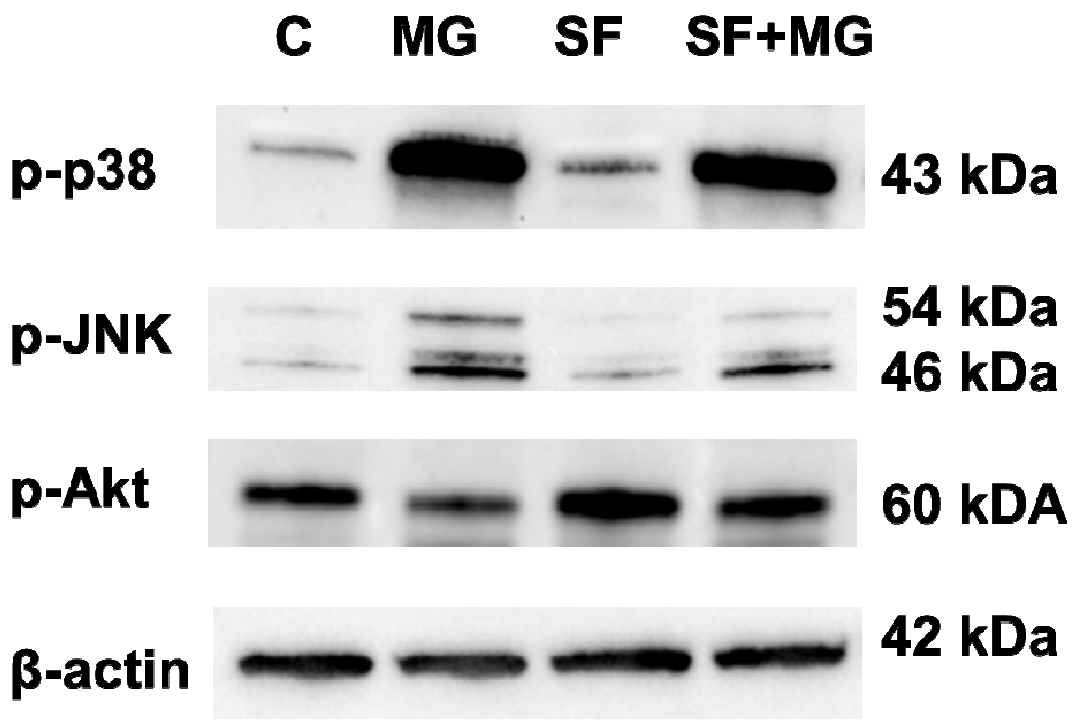


Figure 7-27: SF counteracts MG-induced protein kinase negative modulation. *Cells were pre-treated with 5μM SF and after exposed to 0.5 mM MG for 24 h. Cell lysates were immunoblotted with antibodies for p-p38, p-JNK and p-Akt.*

CHAPTER 8

8. Discussion

In recent years the incidence of chronic degenerative diseases has increased. These diseases have a sneaky and inexorably progressive course that is brought to light when the harm to the patient is already in an advanced stage precluding, in almost all cases, the possibility of an effective treatment that is not only symptomatic. Among them, ischemic heart disease represents the leading cause of death and disability in the Western world, and projections indicate that this record will retain at least until 2030. Great attention has been direct to the identification of novel therapeutic and preventive strategies to counteract the deleterious effects induced by ischemic heart disease. Although nowadays available therapies are good, morbidity and mortality are still very high. Recently there has been considerable improvement in the treatment of acute ischemic heart disease through procedures that enable the rapid restoration of blood flow to the affected area of the myocardium. Such treatment is also known as reperfusion therapy. However, even if blood flow restoration is essential for the viability of the heart, it induces a burst of ROS generation that exacerbates ischemic injury. From this point of view, a promising therapeutic strategies is ischemic preconditioning (PC) that

is recognized as one of the most potent protective mechanisms against myocardial ischemic injury. Ischemic PC is a phenomenon in which a brief exposure to ischemia renders the heart more resistant to a subsequent sustained ischemic insult. Many studies (Schulz et al. 2004) have supported the hypothesis that ROS might play an important role both in ischemic/reperfusion injury and ischemic PC. In this thesis, we clearly observed this paradoxical effect; in fact, 100 μM H_2O_2 were able to counteract or exacerbated oxidative stress depending on the exposure time: 10 min led to a protective pattern, whereas 30 min caused a marked cell death. Moreover, we measured a substantial reduction of intracellular ROS production in H_2O_2 PC treated cardiomyocytes, postulating that H_2O_2 PC elicits its cardioprotective effect through the modulation of the intracellular antioxidant defense system. The capacity of cells to maintain cellular homeostasis during oxidative stress resides in rapid activation or induction of protective enzymes, which in turn decrease oxidative stress by reducing ROS. Interestingly, H_2O_2 PC was able to induce fundamental antioxidant and phase II enzymes like NQO1, GR, TR and CAT but failed to induce GST, SOD and GPX. This disjointed regulation was unexpected, as it has been reported that all these enzymes are regulated by the same transcription factor Nrf2 (Brigelius-Flohe et al. 2006; Purdom-Dickinson et al. 2007; Zhu et al. 2008). In agreement with these findings, we observed a marked translocation of Nrf2 to the nucleus immediately after H_2O_2 PC induction and its up-regulation after 6 h from H_2O_2 PC. To better clarify this point we evaluated the effect of H_2O_2 PC on another

transcription factor belonging the same Nrf2 Cap'n'Collar transcription factorfamily: Nrf1. Although Nrf2 has been shown to be crucial in the activation of genes regulated by the antioxidant responsive element (ARE), the involvement of Nrf1 in ARE function has also been demonstrated through transfection studies and gene expression analysis in knockout cells and animals (Biswas et al. 2009). Gene targets of Nrf1 include genes encoding enzymes involved in GSH biosynthesis and other oxidative defence enzymes (Biswas et al. 2009). Our data demonstrated that H_2O_2 PC led to a marked translocation of Nrf1 to the nucleus. Interestingly, It was observed that Nrf1 has the potential to play an important role in modulating the response to oxidative stress by functioning as a trasdominant repressor of Nrf2-mediated activation of ARE-dependent gene transcription. So we can hypothesize that H_2O_2 PC is able to induce both Nrf2 and Nrf1 and their combined translocation to the nucleus leads to this particular pattern of phase II enzymes activation. Further studies will be necessary to clarify this point. The role of MAPKs in PC signal transduction has been addressed by manyAuthors, but only few studies have focused on the role of H_2O_2 in PC signal pathways and data are not conclusive (Nakano et al. 2000; Yue et al. 2000; Hausenloy et al. 2006). It is commonly acceptedthat phosphorylation of ERK 1/2 in cardiac myocytes during early reperfusion serves as a defence mechanism against ischemic stress stimuli (Yue et al. 2000). The role of ERK 1/2 as a potential mediator of protection in the setting of PC has been controversial, with some studies supporting its role (de Silva 2004; Gong et al. 2004) and several studies failing to

demonstrate a role of ERK 1/2 (Mockridge et al. 2000; Mocanu et al. 2002; Button et al. 2005). We observed that ERK 1/2 is markedly activated immediately after H_2O_2 PC and this activation occurred also with not cytoprotective H_2O_2 concentrations. Inhibition of ERK 1/2 by PD did not influence H_2O_2 PC cardioprotection against a subsequent oxidative stress, indicating that ERK 1/2 is probably not involved in this mechanism. Our data are in agreement with the result of Mockridge et al. (2000) that observed a rapid, transient phosphorylation of ERK 1/2 after PC, but ERK 1/2 blocked by PD did not effect cytoprotection elicited by PC. Mocanu et al. (2002) demonstrated that ERK 1/2 cascade is not implicated in ischemic PC isolated perfused rat hearts. The role of p38 MAPK in the protection provided by PC remains controversial. Many studies confirmed role of p38 MAPK as a potential mediator of PC-induced cardioprotection, while others have shown that pharmacologically inhibition of p38 MAPK during ischemia-reperfusion confers cardioprotection in non-preconditioned hearts (these discrepancies are reviewed in Hausenloy and Yellon, 2006). Bell et al. (2008) observed that these different views about the role of p38 MAPK in mediating PC could be ascribed to an inappropriate use of its pharmacological inhibitor SB. In particular, they attributed to DMSO, the common SB vehicle, antioxidant properties that can protect also non-preconditioned hearts. In our study H_2O_2 PC was found to induce a significant increase of p38 MAPK phosphorylation and, in the presence of SB, the beneficial effect of H_2O_2 PC was ablated. To ascertain the influence of DMSO, this compound was added also in control and stressed cells evidencing

no effects. Activation of the PI3K/AKT pathway has been demonstrated to play a key role in both early and delayed myocardial PC, contributing to the recruitment of multiple endogenous cardioprotective pathways to reduce myocardial damage after ischemia and reperfusion (Hausenloy et al. 2005; Hausenloy et al. 2006). In this present studywe demonstrated that H_2O_2 PC induced a significant Akt phosphorylation, and, more important, the cardioprotective effect of H_2O_2 PC was significantly attenuated by the inhibition of PI3K/Akt. Tong et al. (2000) demonstrated that ischemic PC protects the heart by activating the PI3/Akt cascade during the PC protocol, a finding confirmed by subsequent studies. For example, Uchiyama et al. (2004) observed that in adult cardiomyocytes the antiapoptotic effect of PC against hypoxia/reoxygenation requires Akt signaling leading to phosphorrylation of BAD, inhibition of cytocrome c release, and prevention of caspase activation. Moreover, Mocanu et al. (2002)demonstrated that wortmannin and LY (PI3 kinase inhibitors) partially blocked PC cardioprotection in isolated perfused rat hearts. Many Authors have demonstrated the involvement of p38 MAPK and Akt in Nrf2 activation (Zipper et al. 2000; Owuor et al. 2002; Kim et al. 2009), but no studies have been undertaken to demonstrate their role in the induction of phase II enzymes in cardiomyocytes. Here we report that inhibition of both p38 MAPK and Akt in H_2O_2 PC significantly reduced protein expression and activity of GR, Tred and CAT while did not modify NQO1, in agreement with data obtained by Manandhar et al. (2007) on murine keratinocytes in which the pharmacological inhibitors of p38 MAPK,

PI3K/Akt, and PKC did not alter 3H-1,2-dithiole-3-thione induction of NQO1.

As it has been widely emphatized in this thesis, more and more studies evidencing a role of carbonyl stress, as well as oxidative stress, in the development of chronic degenerative diseases. The main reactive carbonyl species present in physiological systems, responsible for glycation, is methylglyoxal (MG). The enzymatic defenses against MG-induced glycation consist of 2 enzymes, GLO1 and GLO2 , that forms the glyoxalase system. Recently, it has been observed that GLO1 expression is regulated by Nrf2/ARE pathway. Between the substances able to modulate this pathway, great attention has been dedicated to the natural compound SF, derived from glocoraphanine. Based on this observations further aim of this thesis was to study the effect of SF in counteracting glycative MG-induced damage in cardiomyocytes, moreover, for this time, we demonstrated that SF counteracted MG-induced apoptotic cell death, reduced ROS generation and AGE production, and induced GLO1 expression and functional activity. Likely, SF elicits a multi-target behavior against MG-induced damage: it up-regulates GLO1 enhancing the cell ability to detoxify MG, and acts as an indirect antioxidant reducing ROS produced by MG. Even if the exact role of the glyoxalase system in cardiovascular disease has not been fully elucidated, it has been observed that in hemodialysis patients, the GLO1 A491C polymorphism is associated with a significantly higher prevalence of cardiovascular disease and peripheral vascular disease risk (Kalousova et al. 2008). Moreover, Wang et al. (2010) demonstrated that

thioredoxin inactivation and significantly protected cardiomyocytes from I/R injury. These results suggest that any intervention able to reduce intracellular MG concentration, like GLO1 induction by SF treatment, could attenuate injury endured in myocardial I/R processes. More and more scientific evidences are confirming a role of glycation also in the onset of diseases such as Alzheimer's disease (Luth et al. 2005). AGEs are able to cross-link proteins in Aβ deposits and neurofilamentes AD level leanding to tau hyperphosphorylation. It has also been observed that MG is a neurotoxic mediators of oxidative stress in the progression of AD inducing apoptosis and cellular dysfunction. For these reasons it can be reasonably hypothesized that AD might be strongly linked to an increase in MG levels due to an oxoaldehyde detoxification impairment or an altered endogenous oxoaldehyde production. Since we have demonstrated that SF is able to counteract MG-induced damage in cardiomyocytes, last aim ofthis thesis was to study the ability of SF to counteract glycation in SHSY-5Y neuroblastoma cell line. The human SHSY-5Y neuroblastoma cell line is well characterized and has been widely used as a model system to investigate the pathological effects of various neurotoxic compounds. In addition, SHSY-5Y cell costitutively express a receptor for AGEs. They also are highly sensitive to MG challenge due to a defect in thei antioxidant and detoxifying abilities that prevent efficient scavenging and that elicits extensive caspase -9-dependent apoptosis (Li et al. 2011). As already observed, MG accumulation is responsible for detrimental effects on neurons viability (Di Loreto et al. 2004; Di Loreto et al. 2008). Our data

demonstrated that SHSY-5Y death occurs through the induction of ROS-mediated cell death. Indeed, MG was as able to increase intracellular ROS level and to decrease reduced GSH. SF treatment was able to counteract Mg triggered cell death and to reduce ROS production. MAPK signaling pathways are activated by many extracellular stimuli such as cytokines, growth factors and oxidative stress (Seger et al. 1995). It has been indicated that MAPK-signaling cascades are involved in the process of apoptosis (Cross et al. 2000). In our study, MG was able to phosphorylate the pro-apoptotic kinases p38 MAPK and JNK at already 30 min from MG exposure and to reduce the phosphorylation of the anti-apoptotic kinase Akt at longer exposure time. Our data are in agreement with the results of Huang et al. (2008)that observed an increased phosphorylation of JNK and p38 MAPK after 400 μM MG treatment in neuro-2A cells. Interestingly, our data demonstrate that SF treatment is able to revert this pro-apoptotic MAPK modulation, confirming its ability to counteract MG induced apoptosis. Since we have previously demonstrated that SF is able to significantly increase GLO1 protein level and activity in cardiomyocytes, we tested its effect on GLO1 regulation also n SHSY-5Y neuroblastoma cell line. In agreement with the dataobtained in cardiomyocytes, Sf increases GLO1activity demonstrating its pleiotropic effect in counteracting MG-induced damage: SF is able to counteract MG-induced apoptosis both modulating fundamental kinases involved in pro-apoptotic signaling cascades and enhancing MG detoxifying system.

In conclusion this thesis show that it is possible to counteract the oxidative and carbonyl stress using different strategies. To one side our data demonstrate that the protection enduced by H_2O_2 is mediated by detoxifying and antioxidant phase II enzymes induction, regulated, not only by transcriptional factor Nrf2, but also by Nrf1. These novel data contribute to our understanding of the mechanisms of H_2O_2 in triggering ischemic PC, opening new researches on the molecular mechanisms at the base of the cardioprotection induced by preconditioning. On the other side our data represent an innovative result because for the first time it was demonstrated the possibility of inducing GLO1 by SF supplementation. This strategy represent an efficient approach for the protection from oxidative and carbonyl stress. These result pave the way to new and more important studies because glycation in involved not only in cardiovascular diseases or in Alzheimer pathology but also in other important chronic degenerative diseases, in ageing, in diabetes, therefore the SF supplementation could represent an efficiency protective/preventive strategy.

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