UNIVERSITÀ DEGLI STUDI DI MESSINA

DIPARTIMENTO DI MEDICINA CLINICA E SPERIMENTALE

XXIX CICLO DI DOTTORATO IN SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI

REACTIVE OXYGEN SPECIES AND CELL INJURY

TESI DI DOTTORATO:
Dott.ssa Federica GALFO

RELATORE:
Ch.mo Prof. Francesco Squadrito

CO-RELATORE:
Prof.ssa Alessandra Bitto

ANNO ACCADEMICO 2015/2016

Faber est suae quisque fortunae

Appio Claudio Cieco

Table of contents

Chapter III:

Chapter I

Reactive Oxygen Species (ROS): overview of ROS involvement in disease pathogenesis

Introduction

Reactive oxygen species (ROS), such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds. When ROS overwhelm the cellular antioxidant defense system, whether through an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and has been implicated in cancer (*Trachootham D, et al., 2009*), neurodegeneration (*Redza-Dutordoir M, et al., 2016*; *Shukla V, et al., 2011*), atherosclerosis, diabetes (*Paravicini TM et al., 2006*), and aging (*Haigis M et al., 2010*). However, ROS involvement in the pathogenesis of disease states is not confined to macromolecular damage. There is increasing

evidence that ROS signaling contributes to disease. For example, ROS have been shown to promote tumor metastasis through gene activation (*Ishikawa K et al., Science 2008*). While there exists ample evidence demonstrating the role of ROS in regulating cellular signaling pathways, the question that is raised is exactly how do ROS initiate cellular signaling? The "oxidative interface" is that boundary between ROS and the signaling molecules they activate; that is, the figurative region that describes how ROS directly activate oxidative stress-responsive pathways (Fig. 1).

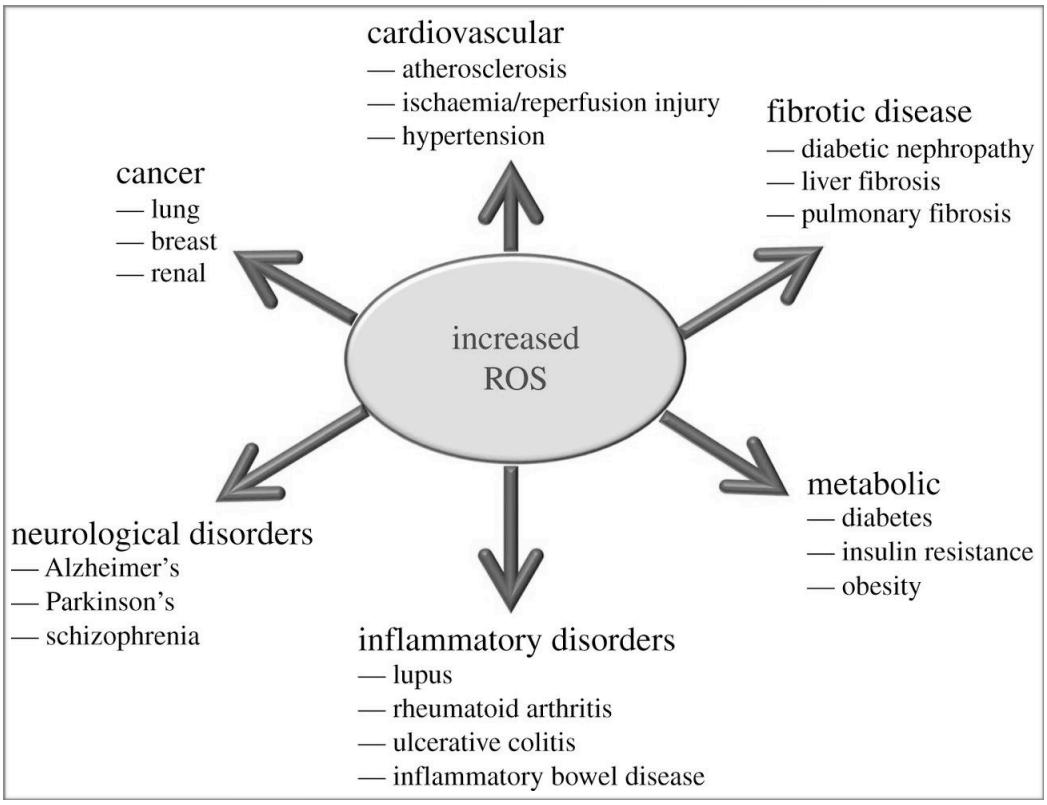

Figure 1: ROS involvement in disease pathogenesis. Schematic representation of the effects of increased ROS production in the development of various pathologies.

We know that the balance between ROS is tightly regulated and extremely important for maintaining vital cellular and biochemical functions. This balance, often referred to as the redox potential, is specific for each organelle and biological site, and any interference of the balance in any direction might be deleterious for the cell and organism. Changing the balance towards an increase in the pro-oxidant over the capacity of the antioxidant is defined as oxidative stress and might lead to oxidative damage. Changing the balance towards an increase in the reducing power, or the antioxidant, might also cause damage and can be defined as reductive stress.

Superoxide Ion Radical, (O^{2-}/HO^{2})

Because most radicals are short-lived species, they react quickly with other molecules. Some of the oxygen-derived radicals are extremely reactive with a short half-life. For example, OH can survive for 10^{-10} sec in biological systems. Non-radical metabolites also possess a relatively short half-life varying from parts of seconds to hours, as in the case of HClO. Obviously, the physiological environment, consisting of such factors as pH and the presence of other species, has a great influence on the half-life of ROS.

Toxicity is not necessarily correlated with reactivity. In many cases a longer half-life of a species might imply a higher toxicity of the compound by allowing it adequate time to diffuse and reach a sensitive location where it can interact and cause damage a long distance from its site of production. For example, the relatively long half-life of superoxide radicals permits them to move to locations where they can undergo interaction with other molecules; these radicals can be produced in the mitochondrial membrane, diffuse towards the mitochondrial genome, and reduce transition metals bound to the genome. On the other hand, a highly reactive species with an extremely short life span, like OH, is produced in locations where it can cause damage (*Barnese K et al., 1981*) by interacting with its immediate surroundings. If there is no essential biological target adjacent their production site, radicals will not cause oxidative damage. The high reactivity of radicals and their short life span illustrate the potential toxic effect and difficulties in preventing oxidative damage. To prevent the interaction between radicals and biological targets, the antioxidant should be present at the location

where the radicals are being produced in order to compete with the radical for the biological substrate.

The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O2^{-}$), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions.

Dismutation of $O2^{-}$ (either spontaneously or through a reaction catalysed by superoxide dismutases) produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH^{-}), one of the strongest oxidants in nature. The formation of OH^{-} is catalysed by reduced transition metals, which in turn may be re-reduced by $O2^{-}$, propagating this process (*Liochev & Fridovich, 1999*). In addition, $O2^{-}$ may react with other radicals including nitric oxide (NO^{-}) in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is also a very powerful oxidant (*Beckman & Koppenol, 1996*; *Radi R et al. 2002*).

In vivo, $O2^{-}$ is produced both enzymatically and non-enzymatically. Enzymatic sources include NADPH oxidases located on the cell membrane of polymorphonuclear cells, macrophages and endothelial

cells (*Babior, 2000*; *Vignais, 2002*; *Babior et al. 2002*) and cytochrome P450-dependent oxygenases (*Coon et al. 1992*). The proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source of both $O2^{-}$ and H_2O_2 (and therefore constitutes a source of OH^{-}) and has been proposed to mediate deleterious processes *in vivo*.

The non-enzymatic production of $O2^{-}$ occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (for example, flavins or iron sulfur clusters) or by xenobiotics previously reduced by certain enzymes (for example, the anticancer agent adriamycin or the herbicide paraquat).

The mitochondrial electron transport chain contains several redox centres that may leak electrons to oxygen, constituting the primary source of $O2^{-}$ in most tissues.

Mitochondrial ROS production

Mitochondria are an important source of ROS within most mammalian cells. This ROS production contributes to mitochondrial damage in a range of pathologies and is also important in redox

signaling from the organelle to the rest of the cell. Consequently, knowledge of how mitochondria produce ROS is vital to understand a range of currently important biomedical topics (Fig. 2).

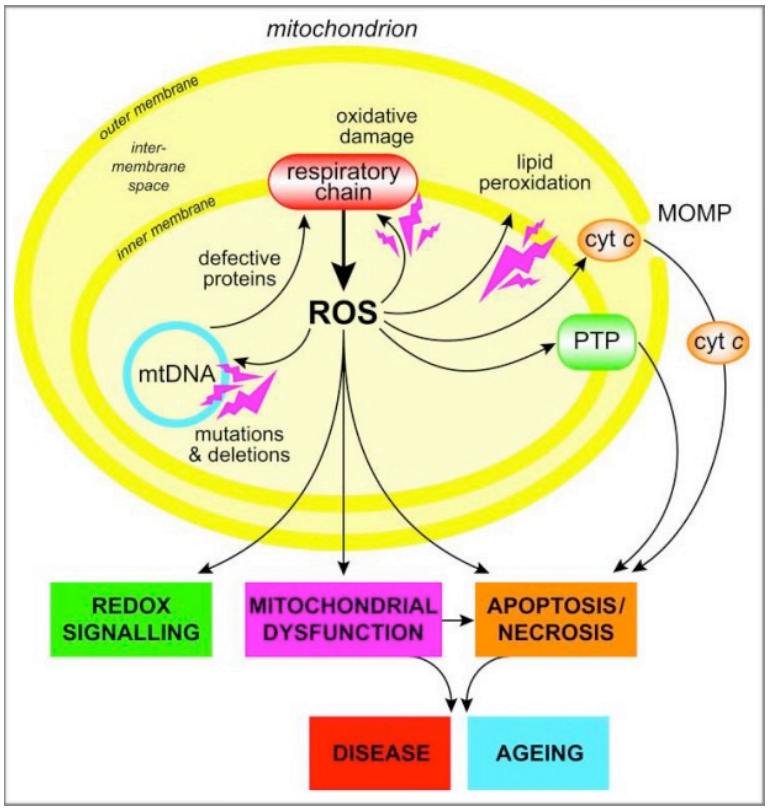

Figure 2: **Overview of mitochondrial ROS production**

ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions. In addition, mitochondrial ROS production leads to induction of the mitochondrial permeability transition pore (PTP), which renders the inner membrane permeable to small molecules in situations such as ischaemia/reperfusion injury.

Mitochondria are double-membrane organelles involved in numerous cellular processes like apoptosis induction, reactive oxygen species generation, adaptive thermogenesis, ion homeostasis and innate immune responses (*W.J.H. Koopman, et al., 2012*). Classically, mitochondria also act as key suppliers of cellular energy by providing the machinery that generates ATP from the energy stored in NADH and $FADH_2$. The latter two molecules are generated by the glycolysis pathway in the cytosol (NADH) and the tricarboxylic acid (TCA) cycle in the mitochondria (NADH and $FADH_2$), and are oxidized at complex I (CI) and complex II (CII) of the Electron Transport Chain (ETC), respectively. The factors determining the rate of $O2^{-}$ production by mitochondria are relatively straightforward. The first is the concentration of the enzyme or protein [E] containing electron carriers that can exist in a redox form able to react with O_2 to form $O2^{-}$. The second is the proportion (PR) of this enzyme's electron carrier present in a redox form that can react with O2. As many redox-active groups exist only transiently in a state that can react with O_2, PR is a time-average. The remaining factors are the local [$O2^{-}$] and the second-order rate constant (kE) for the reaction of that electron carrier with O2 to form $O2^{-}$.

This can be extended to consider several potential electron- donor sites within mitochondria, and also to take into account multiple electron donor sites within a single protein. The concentration of the enzyme responsible for $O2^{-}$ production, [E], will vary with organism, tissue, state, age or hormonal status, and may underlie many of the changes in maximum ROS production capacity between tissues (*Barja, G. 1999*); for example, complex I content may explain the different maximum capacities of pigeon and rat heart mitochondria.

Mitochondrial Electron Transport Chain

The mitochondrial electron transport chain (ETC) has been recognized as one of the major cellular generators of reactive oxygen species (ROS), which include superoxide (O_2), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (OH^-; *Loschen et al. 1971; Boveris et al. 1972; Chance et al. 1979*). The released electrons are then transported to complex III (CIII) by coenzyme Q (CoQ) and subsequently to complex IV (CIV) by cytochrome *c* (cyt *c*). At the latter complex, the electrons react with molecular oxygen (O_2) to form water. Electron transport is energetically coupled to the translocation of protons from the mitochondrial matrix across the mitochondrial

inner membrane (MIM) at CI, CIII and CIV. This results in an inward-directed proton-motive force (PMF) across the MIM that consists of a chemical (ΔpH) and an inside-negative electrical gradient (Δψ).

Protons are allowed to flow back into the mitochondrial matrix *via* the F_oF_1-ATP-synthase (CV) to drive the synthesis of ATP from ADP and inorganic phosphate (P_i). Together with CV, the four complexes of the ETC constitute the oxidative phosphorylation (OXPHOS) system. By reverse-mode action CV can also hydrolyse ATP and expel protons from the mitochondrial matrix thereby sustaining Δψ (Fig. 3) (*D.G.* Nicholls et al.,1972).

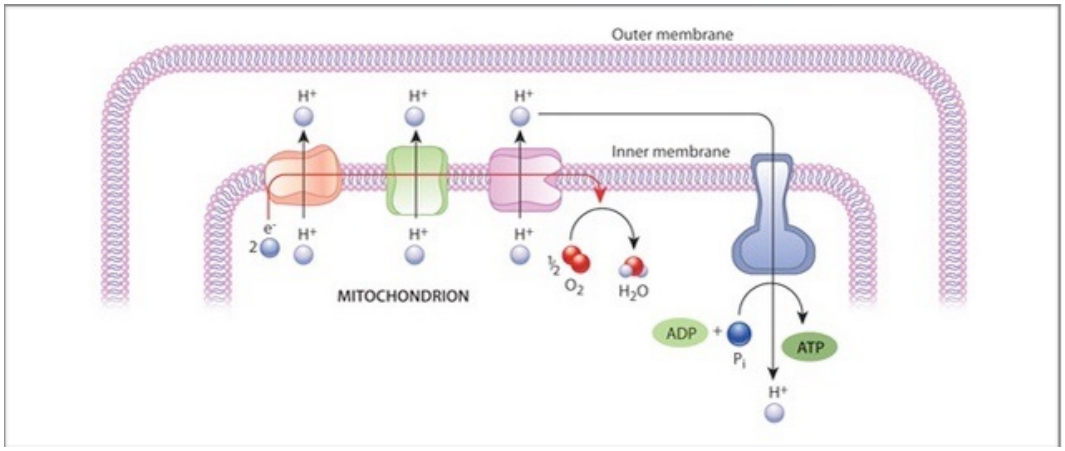

Figure 3: At the inner mitochondrial membrane, a high energy electron is passed along an electron transport chain. The energy released pumps hydrogen out of the matrix space. The gradient created by this drives hydrogen back through the membrane, through ATP synthase. As this happens, the enzymatic activity of ATP synthase synthesiszes ATP from ADP.

This mechanism requires that ATP is transported from the cytosol to the mitochondrial matrix by reverse-mode action of the mitochondrial adenosine nucleotide translocase (ANT) or is generated by mitochondrial substrate-level phosphorylation (*C. Chinopoulos 2011*). A sufficiently large PMF is not only required to sustain ATP production but also is of crucial importance for mitochondrial fusion, protein import, metabolite exchange with the cytosol, and apoptosis induction (*F. Palmieri 2008, F. Fischer et al., 2012, J. Nunnari et al., 2012 and E.I. Rugarli e al., 2012*). The proton pump of the electron transport chain (ETC) or mitochondrial respiratory chain establishes the mitochondrial membrane potential or proton motive force (Δp). The protons are pumped from the matrix space to the intermembrane space, creating a substantial pH and electrical gradient across the mitochondrial inner membrane.

These protons eventually re-enter the matrix space via the ATPase, driving the synthesis of ATP. This potential is dependent on a variety of other mitochondrial functions and is involved in the apoptotic process. In this sense, $\Delta\psi$ can be considered as an important functional readout of mitochondrial health.

In addition to being the source of energy that supports life under aerobic conditions, mitochondria can also be the source of signals that initiate apoptotic cell death (*Gottlieb RA, 2000*).

Mitochondria contain key regulators of caspases; a family of proteases that are major factors in many apoptotic processes (*Wang X, 2001*).

Cytochrome *c* is released from the mitochondrial intermembrane space inducing the assembly of the apoptosome that is required for activating downstream caspases. Cytochrome *c* release from mitochondria is a key event in initiating apoptosis, but the actual mechanism of its release is still debatable. In particular, the relation between mitochondrial physiology and the release of cytochrome *c* and other apoptogenic factors from mitochondria is not clear. Mitochondria utilize oxidizable substrates to produce a membrane potential in the form of a proton gradient across the mitochondrial inner membrane. It was shown that the supply of oxidizable substrates to mitochondria depends on the concentration of external growth factors (*Vander Heiden MG et al., 2001*). Withdrawal of growth factors or loss of the extracellular glucose supply will lead to a decline in mitochondrial membrane potential (MMP). If growth factor

or glucose deprivation persists, cells ultimately undergo apoptosis that is initiated by cytochrome *c* release from mitochondria.

Whether changes in mitochondrial physiology contribute to the initiation of cell death in response to growth factor withdrawal remains a controversial issue.

The mitogen-activated protein kinase (MAPK) cascades consist of four major MAPKs; the extracellular signal-related kinases (Erk1/2), the c-Jun N-terminal kinases (JNK), the p38 kinase (p38), and the big MAP kinase 1 (BMK1/Erk5). These kinases are evolutionarily conserved in eukaryotes and play pivotal roles in cellular responses to a wide variety of signals elicited by growth factors, hormones, and cytokines, in addition to genotoxic and oxidative stressors. Function and regulation of the MAPK cascades have been comprehensively covered (*Raman M et al., 2007; Cuadrado A et al., 2010; Weston CR et al., 2007; Ramos JW et al., 2008*).

Among the members of the MAPK cascades, apoptosis signal-regulated kinase 1 (ASK1) is an upstream MAPKKK that regulates the JNK and p38 MAPK pathways leading to apoptosis through phosphorylation of MKK4, MKK3, and MKK6 MAPKKs (*Ichijo H et al., 1997*). ASK1, once activated under various stress conditions including

oxidative stress (*Tobiume K et al., 2001*), is homo-oligomerized by both C- and N-terminal coiled-coil domain interaction and activation occurs through phosphorylation of a conserved threonine (Human: Thr-838, Mouse: Thr-845) residue in the activation loop of the human ASK1 kinase domain. ASK1-deficient mouse embryonic fibroblasts were shown to be less susceptible to TNF or H_2O_2-induced cytotoxicity along with decreased JNK and p38 MAPK activation, suggesting that ASK1 plays a pivotal role in promoting cell death under oxidative stress; however, ROS activated ASK1 mediates p38 signaling leading to non-apoptotic outcomes also, such as differentiation (*Takeda K et al., 2007*) and immune signaling (*Matsuzawa A et al., 2005*), thus reinforcing the role of ROS signaling in cellular homeostasis.

Oxidative stress- induced cell death

Apoptosis plays an essential role in normal development and tissue homeostasis, and when dysregulated it contributes to various diseases including cancer, autoimmunity and neurodegenerative disorders. There is a direct interaction between ROS levels and induction of cell death and mitochondria play a central decision-

making role in executing the cell-death program. The liberation of toxic factors together with the identification of key regulatory proteins that influence mitochondrial function, each suggesting that the mitochondrion may be an important therapeutic target for the design of new interventions to abrogate or at least mitigate inordinate cell death after burn. How burn signals become integrated at the level of the mitochondria to activate the cell-death pathway is unknown and is an area of active investigation.

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) family member that activates the JNK and p38 MAP kinase pathways and is activated by various stresses including oxidative stress, TNFα, calcium overload, and endoplasmic reticulum stress.

Recent analyses of ASK1-deficient mice have revealed that ASK1 is required for cell death induced by oxidative stress, TNFα, and endoplasmic reticulum stress (*Guo X et al., 2010*).

Oxidants have been demonstrated to regulate the activation of c-Jun N-terminal kinase (JNK), as well as the transcription factor NF-κB.

JNK is a member of the family of mitogen-activated protein kinases, which is well known to be activated by oxidants and a

variety of other stresses in many cell types. The contribution of JNK to many phenotypic outcomes, including survival (*J Wang, et al., 2011*) and apoptosis (*Li D, et al., 2015*), appears to depend upon the cell type, stimulus, the duration of JNK activation as well as the engagement of other signaling modules (*JW Chambers, et al., 2011*).

ROS activation of JNK can induce extrinsic or intrinsic apoptotic signaling (*Win S et al., 2014*). Upstream of JNK is the redox sensitive MAPK kinase kinase, ASK1. ASK1 activity is inhibited by interactions with redox proteins (Grx, Trx1), heat shock proteins (Hsp90, Hsp72) (Fig. 4). TNFα is a potent activator of MAPK cascade, and the ASK1-JNK pathway plays an important role in TNF-R1-mediated apoptotic signaling in various cell types.

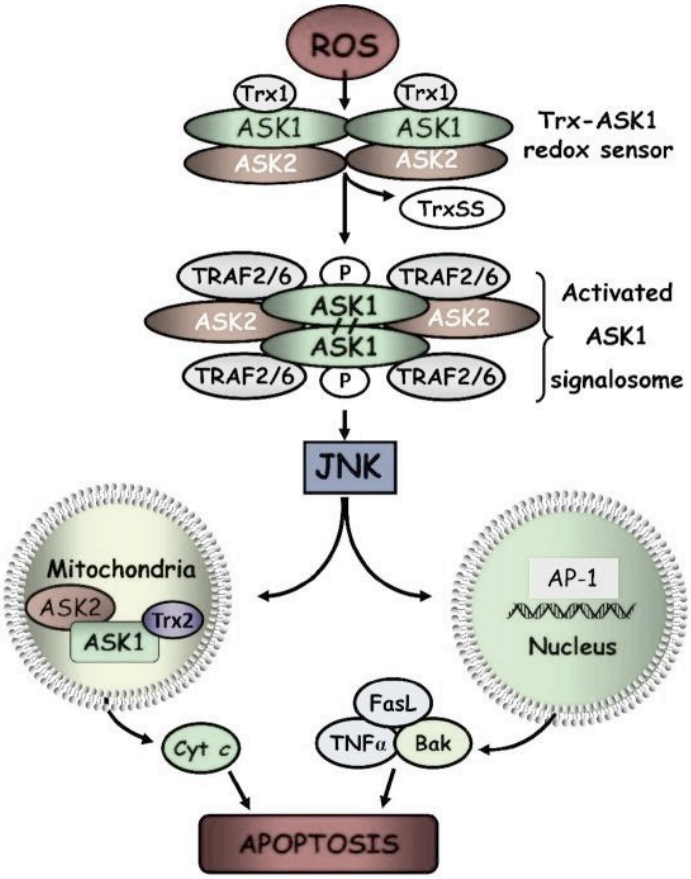

Figure 4: Summary our current understanding of the mechanism of ROS and redox modulation of ASK1/JNK signaling in cell apoptosis. This model proposes that ROS mediates the interaction of Trx1 and the N-terminal domain of ASK1, preventing ASK1 activation and downstream propagation of an apoptotic signal (Magdalena L.Circu, 2010).

Contribution of ROS in burn

During the early phase of a burn, significant neutrophil accumulation was demonstrated in various tissues such as gastric mucosa, liver and lung suggesting that the source of ROS could be neutrophils sequestered in systemic organs as a result of the systemic inflammatory reaction to a local burn insult. Neutrophil accumulation

in these tissues following severe burn may be involved in the pathogenesis of remote organ damage by production of ROS. Normally, cells are able to defend themselves from damaging effects of oxygen radicals in the normal physiologic condition by way of their own antioxidant mechanisms including enzyme systems, vitamins, elements, and some antioxidant molecules.

Normally, there is an exquisite balance between production and destruction of ROS. When this equilibrium is destroyed, ROS are produced excessively and all tissues are exposed to oxidative injury. Severe burn is a trauma with high oxidative stress. After burn, generalized tissue inflammation is present in uninjured organs within hours. Evidence from animal models and human studies (*Wiggins-Dholvic et al 2014*) demonstrated involvement of oxidative stress in burn injuries.

After a burn trauma the necrosome formation initiates the cascade of phosphorylation of several downstream target proteins including phospholipase A2, the proteases calpains and cathepsins, the cytoplasmatic NOXA1/NADPH oxidase complex, and the mitochondrial complex I, thereby leading to excessive ROS production, ATP depletion, and opening of the mitochondrial

permeability transition pores. These events are accompanied by prolonged JNK activation and necrosome-induced stimulation of glycolysis, glycogenolysis, and glutaminolysis as well as the stimulation of Krebs cycle.

Contribution of ROS in I/R injury

Oxidative stress has been suggested to be involved in a number of cardiac pathologies including heart failure (*H Tsuitsui et al 2011*). Evidence to support this claim has come from animals models of myocardial infarction, pressure overload and pacing-induced heart failure as well as in patients with chronic left ventricular systolic failure (*Y Azizi et al 2016*). Interestingly, oxidative stress-induced heart failure may be attributed in part to increased cardiomyocyte apoptosis.

Several studies have shown a direct effect between ROS levels and apoptosis. Interestingly, treatment of cardiac cells with H_2O_2 was sufficient to trigger dissipation of inner mitochondrial membrane potential and PTP opening mitochondrial with attendant cytochrome c release and caspase-3 activation.

Importantly, agents that inhibited the PTP opening suppressed H_2O_2-induced apoptosis suggesting that mitochondrial perturbations were fundamental to the apoptotic process.

Recently, apoptosis repressor with CARD domain (ARC), a novel caspase-inhibitory protein restricted to cardiac muscle cells, was shown to block cell death triggered by H_2O_2 in the myogenic cell line, H9c2 (*Neuss M et al 2001*). ARC was also effective in blocking H_2O_2-induced loss of membrane integrity and/or disruption of mitochondrial $\Delta\Psi_m$ in two human cell lines in which it is not normally expressed. These results demonstrate that, in addition to its ability to block caspase-dependent events in apoptosis, ARC also prevents cell death via the preservation of mitochondrial function.

Notably, a study by (Akao et al. 2003) proposed that H_2O_2-induced cardiomyocyte apoptosis is characterized by a specific pattern of mitochondrial defects. The model is defined by three phases: priming, depolarization and fragmentation. During the priming phase, exposure to H_2O_2 stimulates mitochondria to undergo specific changes in inner mitochondrial membrane structure consistent with swelling and loss of cristae ultrastructure. Priming is associated with matrix calcium overload but not alterations in $\Delta\Psi_m$.

In the next phase, aptly named depolarization, myocyte mitochondria undergo a sudden loss in $\Delta\Psi_{m,}$ which is contingent upon PTP opening. Finally, myocytes are dismantled in the fragmentation phase where mitochondria undergo massive swelling and release cytochrome c quickly, and predictably apoptosis ensues. Whether this model applies globally to oxidative stress or to H_2O_2 specifically is undetermined, but it provides a novel perspective on the temporal and spatial relationship of mitochondrial perturbations to cardiomyocyte apoptosis.

Chapter II

Role of NLRP3 inflammasome in multi-organ dysfunction following thermal injury

Introduction

Burns are a serious global health problem, according to the World Health Organization, with over 195,000 related deaths each year. Burn injury alters host immune functions, predisposing patients to opportunistic and nosocomial infections, sepsis, and multiple organ system dysfunction and failure. Burn injury often leads to a systemic inflammatory state, which has been attributed to the resulting exacerbated innate immune response, referred to as systemic inflammatory response syndrome (SIRS; *Hoover L et al 2006; Sauaia A et al 1996*). In addition, the response to the initial burn is often associated with secondary damage to tissues distant from the injured site.

Prolonged exposure to temperatures higher than 40°C leads to denaturation of proteins and finally loss of their plasma membrane integrity. The local changes result in the clinical picture of coagulation and necrosis. Temperature and duration of contact have also a synergistic effect (*Van Heren et al 2013*).

The systemic pathophysiologic changes following thermal injuries affect multiple organs and body systems leading to clinical manifestations including shock, intestinal alterations, respiratory and renal failure, immunosuppression and others. Evidence from animal models and human studies demonstrated the involvement of oxidative stress in burn injuries. Indeed, there is a direct correlation between ROS levels and induction of apoptosis and mitochondria play a central decision-making role in executing the cell-death program.

Acute renal failure is one of the major complications of burns and it is accompanied by a high mortality rate (*Brusselaers, N et al 2010*). Most renal failures occur either immediately after the injury or at a later period. Two major mechanisms are involved in the pathophysiological changes in the kidney: filtration failure and tubular dysfunction caused by various factors and interacting with each other (*Aikawa N et al 1990*). The renal failure that occurs in extensively burned patients is usually associated with failure or dysfunction of other organs in a form of multiple organ dysfunction syndrome which adversely influences the prognosis. Acute renal failure occurring immediately after burns is mostly due to reduced cardiac output, which is mainly caused by fluid loss (*Pruitt BA Jr 2000*).

Many mediators, including cytokines (TNF, IL-1, etc.), prostaglandins (PGs), thromboxane, leukotrienes, and platelet-aggregating (activating) factor (PAF) are produced or released in the early post-burn period. They act variably to increase vascular permeability and to induce tissue damage (*Ansermino M et al 2004*).

An intense affiliation has been demonstrated between the quantity of lipid peroxidation and the degree of burn complications such as remote organ damage and shock. A primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties. The mechanism of this event is the deformation of cell membrane phospholipids by oxidazing radicals.

Cell-death pathway in *burn*

Inflammatory cells produce reactive oxygen species (ROS) as part of the microbiocidal/cytocidal system. Several studies demonstrated that burn initiates systemic inflammatory reactions by producing burn toxin. At molecular level, both complement activation and intravascular stimulation of neutrophils result in the production of cytotoxic ROS. Increased histamine activity, enhanced by the catalytic

properties of xanthine oxidase, causes progressive local increases in vascular permeability.

Activation of c-Jun NH2-terminal kinase (JNK) is based on the production of reactive oxygen species (ROS) and activation of apoptosis signal-regulating kinase-1 (ASK1), a member of the MAP3K family (*Ichijo H et al 1997*).

The pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin -1β (IL-1β) and cell adhesion molecules are overexpressed after severe burns, leading to uncontrolled inflammatory response and organ injury. The excessive production of reactive oxygen species (ROS) can lead to cell damage and finally result in organ failure. Furthermore, ROS overproduction leads to mitochondrial dysfunction and adenosine triphosphate (ATP) depletion (*Dal Pizzol F et al 2010*), triggering cytochrome *c* to leak from the mitochondria into the cytoplasm and ultimately causing cell apoptosis (*Yang Y et al 2015*). Cells are able to defend themselves from damaging effects of oxygen radicals in normal physiological conditions, because the existing balance between production and destruction of ROS. However, how burn signals become integrated at

the level of the mitochondria to activate the cell-death pathway is unknown and is an area of active investigation.

NLRP3 Inflammasome in burn

The activation of NLRP3 inflammasomes has been implicated in a growing number of diverse pathological conditions, ranging from bacterial infections to cardiovascular dysfunction and metabolic syndrome (*Benko S et al., 2008, Duewell et al., 2010, Overley-Adamson et al., 2014*). Despite rapid and extensive efforts in identifying various agents that stimulate the NLRP3 inflammasome, the underlying mechanisms by which these diverse danger signals activate the same molecular machinery remain poorly understood.

ROS, produced by many known activators of NLRP3 inflammasomes, are shown to be a critical mechanism triggering NLRP3 inflammasome formation and activation in response to many exogenous stimuli as well as endogenously produced or secreted molecules from damaged cells such as DAMPs (*Tschopp J et al., 2010*).

Increased production of ROS results from trauma, including burn injury. These oxygen radicals are released from neutrophils after inflammation or ischemic injury. The production of ROS contributes to

increased vascular permeability, tissue edema, systemic inflammation, and multi organ dysfunction.

The hypothesis of ROS as an NLRP3-activating trigger arose when inhibition of NADPH oxidase-derived ROS prevented ATP-induced caspase-1 activation and *IL-1*beta production in alveolar macrophages (*Cruz CM et al., 2007*). Further substantiating this hypothesis, knockdown of the p22phox subunit of NADPH oxidase significantly suppressed IL-1beta release in THP1 cells in response to asbestos and MSU challenge (*Dostert T et al., 2008*).

The crystal structure of NLRP3 contains a highly conserved disulfide bond connecting the PYD domain and the nucleotide-binding site domain, which is highly sensitive to altered redox states (*Bae JY et al., 2011*). The presence of this unexpected disulfide bond between Cys-8 and Cys-108 spans across six species, including humans, monkeys, and mice, and the strict conservation of this bond is indicative of a crucial redox role for NLRP3.

It has been shown that a burn causes a distinct inflammatory cytokine expression profile in severely burned patients (*Finnerty CC et al 2006*) and that the cytokine profile at the time of admission can predict which burned patients will develop infectious complications

and sepsis during the hospital course (*Finnerty CC et al., 2007*). Although these studies enhance our understanding of the post-burn inflammatory response and allow prediction of patient outcome, effective therapies for altering patient outcome do not exist.

In order to investigate new drugs and interventions that will modulate post-burn hypermetabolism and hyperinflammation, genetically modified mice with either altered NLRP3 protein expression (knock-out or knock-in) have been used. Therefore, characterization of the murine inflammatory response to burn is essential to not only allow modulation of the response, but also to allow comparison to the human response to determine the appropriateness of using mice to study the human post-burn response.

Results

Western blotting and histological results

Apoptosis signal-regulating kinase (ASK) 1 is activated in response to various cytotoxic stresses including TNF, Fas and reactive oxygen species (ROS), and activates c-Jun NH2-terminal kinase (JNK) and p38. However, the roles of JNK and p38 signaling pathways during apoptosis have been controversial.

Kidneys from KO animals had detectable expression of ASK1, after 7 days (Fig.1), this expression was instead markedly reduced in KI mice, maybe due to their overexpression of NALP3 that likely processed most of the DAMPs.

The c-Jun NH2-terminal kinase (JNK) is activated when cells are exposed to multiple forms of stress, and this signaling pathway has been implicated as a mediator of stress-induced apoptosis. In this animal model of thermal burn injury, as expected, an high JNK activation has been detected only in KO animals after 7 days (Fig.3).

MAPKs comprise a family of serine/threonine protein kinases that function as critical mediators of signal transduction and include also the p38 MAPKs. As we know, the JNKs and p38 MAPKs are activated

by proinflammatory cytokines and a variety of cellular stresses, including UV light, hyperosmolarity, heat shock, and microtubule disrupting drugs. Because the lack of expression of ASK1 in KI animals, p38 activation in this experimental group might be due to the high inflammatory profile still present after 7 days of being injured compared with WT-injured mice as show by the presence of proptosis in KI animals even under normal conditions (Fig.5).

Bax (member of the proapoptotic group of multidomain Bcl2-related proteins) are essential for the JNK and p38-stimulated release of cytochrome *c* and apoptosis. Indeed, both KO and KI animals present high level of BAX after 7 days as a sign of mitochondrial dysruption and extensive cell death as confirmed by histological results from kidney slices by TUNEL apoptosis assay (Fig.6).

Conclusions

It is generally accepted that burn injury and trauma primes the innate immune system to enhance inflammatory responses to pathogen associated molecular signals (PAMPs), however, the molecular pathways responsible for injury-induced immune system activation are not well defined. The inflammasome was identified as a central feature of the danger response because most of the extracellular or intracellular signals that activate the inflammasome are considered danger molecules or alarmins. A number of theories have been proposed for the identity of the cellular signal responsible for NLRP3 activation, The finding that ER stress, like other NLRP3 activators, activates the NLRP3 inflammasome in a K^+ efflux- and ROS-dependent manner suggests that it may also be sensed by mitochondria. Thus, it is conceivable that ER stress initiates a signal that is transmitted to mitochondria and then relayed to the NLRP3 inflammasome.

Severe thermal injuries result in a wide array of stress-associated inflammatory and metabolic changes aimed at restoring homeostasis of the body. Unfortunately, when these changes become

uncontrolled, persisting far past the initial trauma, they lead to a state of severe metabolic dysfunction.

This study provides evidence of a role of the NLRP3 inflammasome in systemic inflammatory response following thermal injury. Under this peculiar conditions KO-animals displayed an elevated mortality rate (over 60%) during the first 4 days, and none of the KO mice survived after day 7. On the other hand, the animals overexpressing the NLRP3 survived up to 14 days as the WT ones. The excessive triggering of the apoptotic/inflammatory pathways in KI-animals was evident even under normal conditions, in fact pyroptosis was detected in kidneys from sham animals. KO-mice showed an increased activation of apoptosis-related molecules after 7 days, while in KI animals the inflammatory pathway was more activated compared to either WT and KO mice.

These results may suggest that the NLRP3 inflammasome is of fundamental importance in survival and recovery from systemic injury. Indeed, recent studies demonstrated that mitochondrial dysfunction involving increased mitochondrial ROS production (*Galley HF 2011, Zhou et al., 2011*) and the release of mitochondrial DNA into the cytosol are critical events associated with NLRP3 inflammasome

activation. However, Jennifer R. Deuis and colleges demonstrated that NLRP3 and its downstream product caspase-1 have a limited role in the development of acute burn-induced pain and that pharmacological inhibition of NLRP3 is unlikely to be an effective treatment strategy for the treatment of acute procedural pain or inflammation in burn-injured patients. Burned patients often develop also a form of stress-induced diabetes (with hyperglycemia, insulin resistance, and hyperlipidemia) which is linked to marked increases in morbidity and mortality. Particularly, in burned patients, studies have demonstrated that the significant pathophysiological changes and extreme inflammatory responses are not only present during acute hospitalization causing delays in their rehabilitation and reintegration.

Although intensive efforts have long focussed on identifying the underlying mechanisms of these extreme metabolic alterations, few studies have managed to elucidate how thermal injury induces hypermetabolism, prolonged inflammation and stress responses, and insulin resistance, and whether these alterations are responsible for the increased morbidity and mortality (*Stanojcic M et al., 2014*). Others have also suggested that mitochondrial dysfunction and subsequent increases in reactive oxygen species (ROS) may be

responsible for priming and activating the inflammasome (*Sorbara MT et al., 2011; Zhou R et al., 2011*) in metabolic disorders. It would be interesting to investigate this aspect in future research.

Meterial and Methods

Animals

For these experiments we used 3 different strain of mice: the hyper expressing Nlrp3A350VneoR (k-in), the null Nlrp3L351PneoR mice (ko) and their relative wild type strain C57BL/6J. Animals were kept under standard conditions at the Animal Facility of Messina University.

Thermal burn injury

To produce a second degree scald burn, a 6 cm^2 area on the back of each animal was shaved (30% of the body surface). Mice were anesthetized and immersed in 80°C water for 10 seconds; saline solution was administered to allow recover. All animals (n=5 for experimental group) were allocated in single cages and killed after 7 days for further analysis.

Western Blotting analysis

Protein samples from kidney were extracted to detect p-ERK 1/2, p-JNK 2/3, p-p38, p-ASK1 and BAX. Protein samples (30 mg) were

separated in SDS-PAGE and blotted on PVDF membranes. To prove equal loading, the blots were stripped and beta-actin expression assessed.

Histologycal analysis

For light microscopy tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5-um thickness and stained with hematoxylinn and eosin (H&E) or with a TUNEL assay kit (GenScript).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). The significance of difference was assessed by Unpaired T-test. P values less than 0.05 was considered significant. Graphs were performed using GraphPad Prism (version 5.0 for Windows).

LIST OF FIGURES

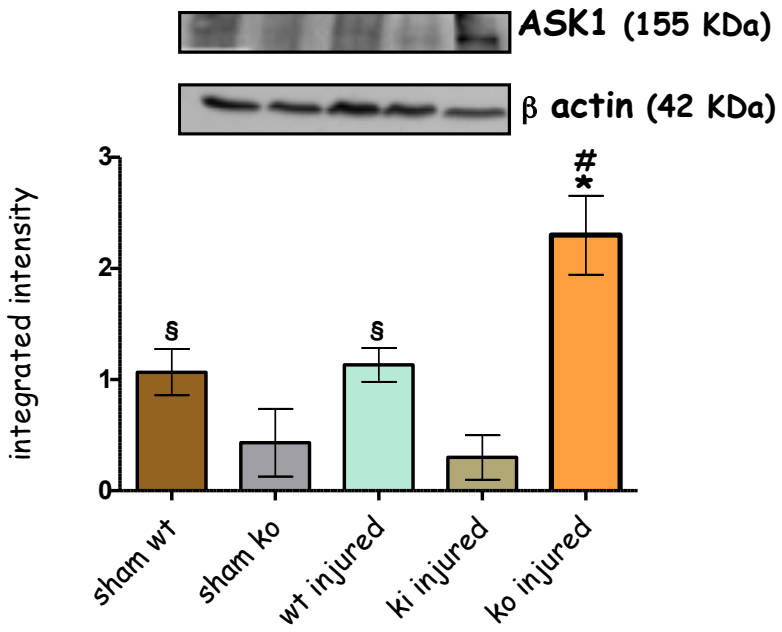

Figure 1: Kidneys levels of ASK1 after 7 days. KO vs KI *p<0.001; KO vs WT-inj #p<0.05; WT-inj vs KI §p=0.02; (Mann-Withney t-test).

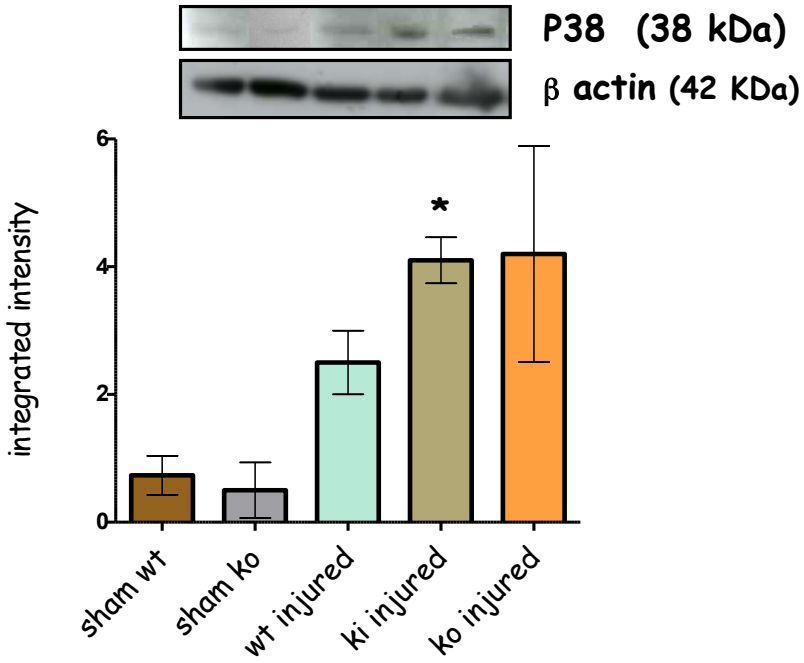

Figure 2: p38 western blot levels in kidneys after 7 days.
KI vs WT-inj *p=0.01 (Mann-Withney t-test).

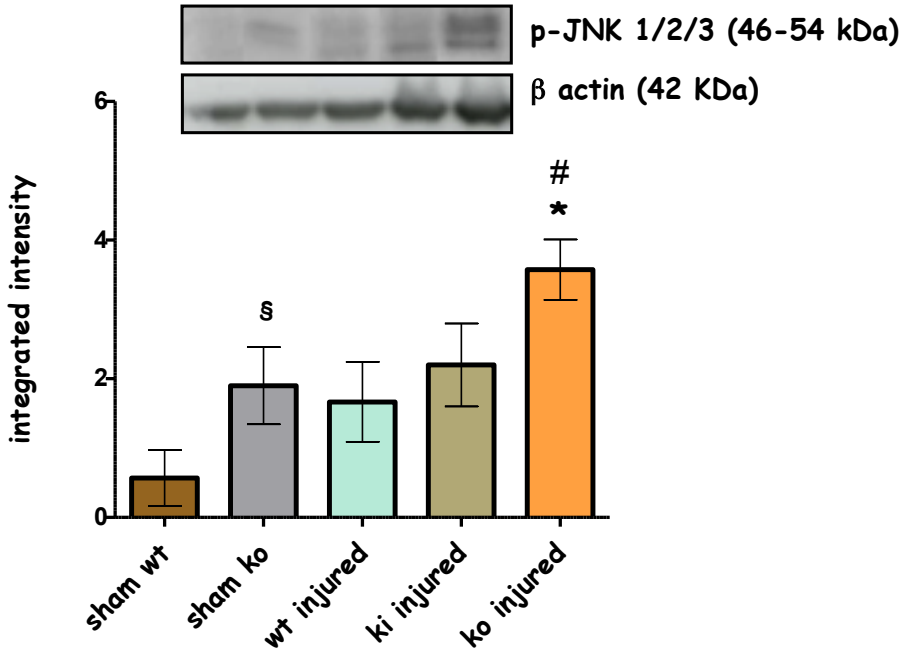

Figure 3: Kidneys levels of JNK after 7 days. KO vs KI *p=0.01; KO vs WT-inj #p<0.005; Sham ko vs Sham wt §p=0.02(Mann-Withney t-test).

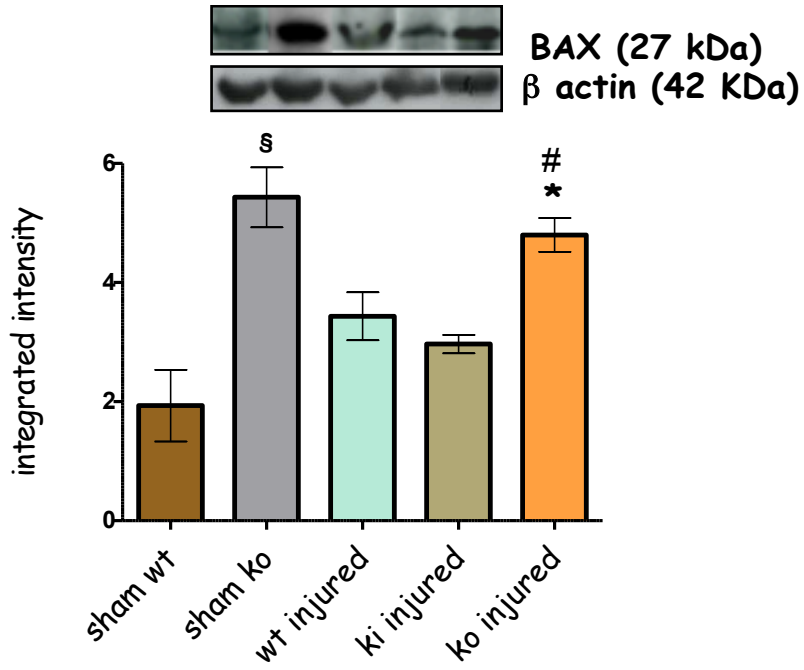

Figure 4: Bax protein levels in kidneys after 7 days confirmed activation of mitochondrial cell death pathway. KO vs KI *p<0.001; KO vs WT-inj #p<0.005; Sham KO vs Sham WT §p<0.001(Mann-Withney t-test).

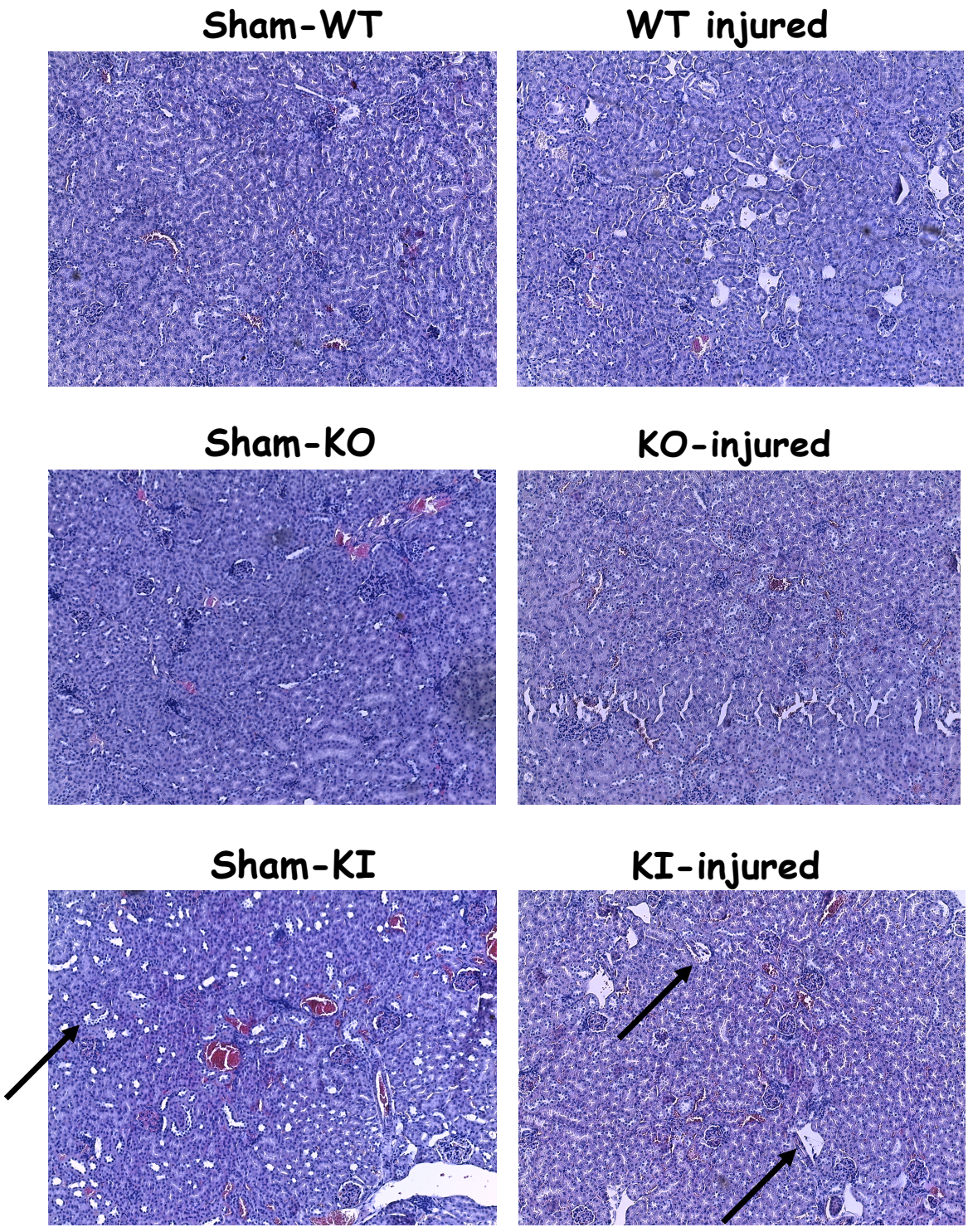

Figure 5: Histological evaluation by H&E of kidneys after 7 days shows an excessive triggering of the inflammatory pathways in KI-animals even under normal conditions, in fact pyroptosis (black arrows) was detected in kidneys from sham animals .

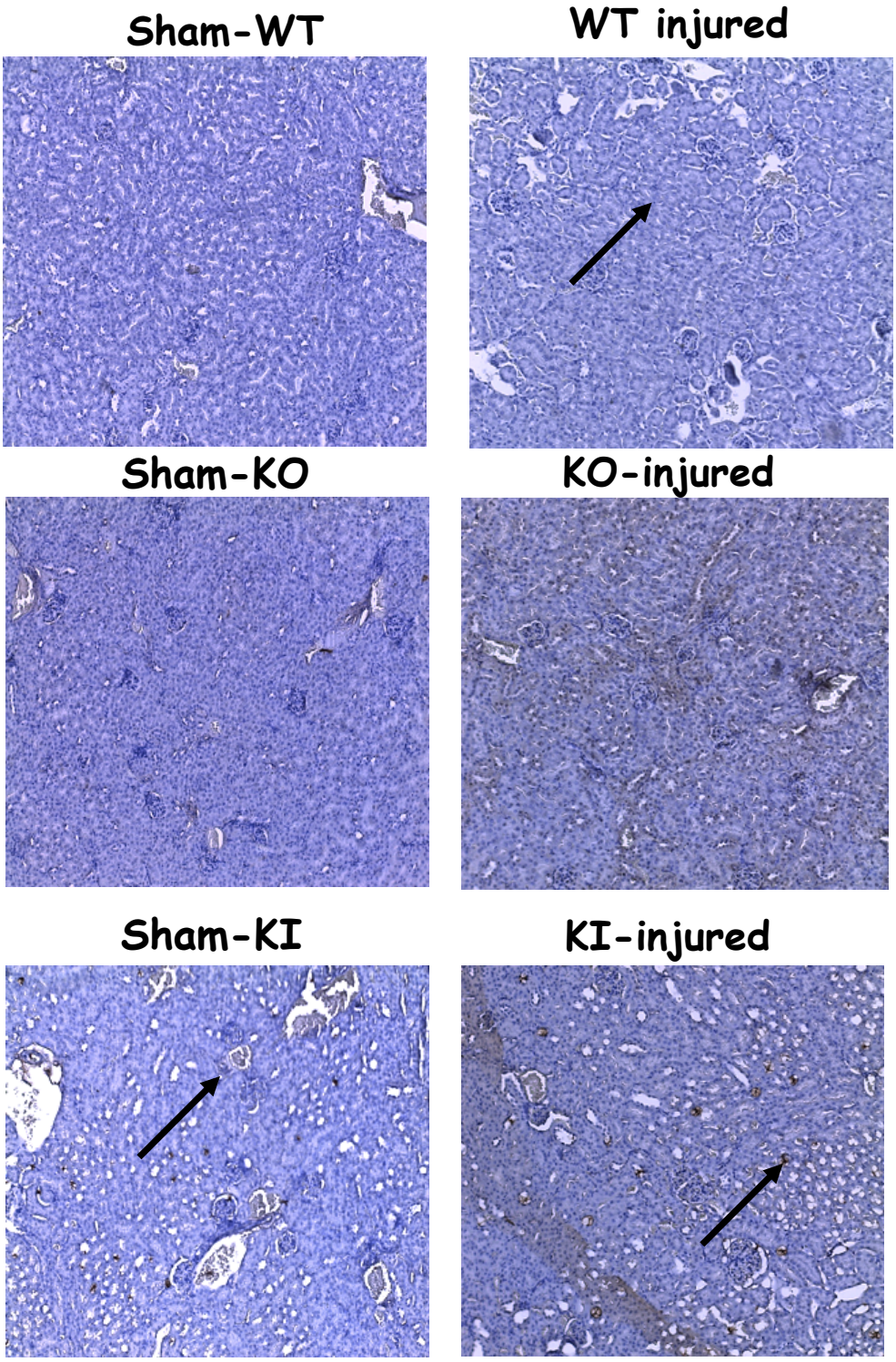

Figure 6: Histological evaluation of kidneys slices by TUNEL-apoptosis-assay shows an increased presence of apoptosis (black arrow) in KO injured animal animals and apoptotic bodies in KI group even under normal condition (black arrow).

Chapter III

Protective effect of Ammonium Tetrathiomolybdate (ATTM) following Ischemia Reperfusion (I/R) Injury

Federica Galfo[1,2], Alex Dyson[1] and Mervyn Singer[1]

[1]Bloomsbury Institute for Intensive Care Medicine,University College London, UK. [2] Department of Clinical and Experimental Medicine, University of Messina, Italy

Introduction

In tissue subjected to ischemia followed by reperfusion (I/R), pathologic mechanisms are elicited that produce reversible cell injury and dysfunction, which can progress to irreversible damage if the nature and extent of ischemia is prolonged or if the pathologic sequelae to reperfusion are of sufficient magnitude. This damage is referred to as I/R injury and can be divided into different phases (*Hausenloy et al 2013, Moens AL et al 2005)* (Fig. 1).

During ischemia (the first phase of injury), interruption of the blood supply to an organ causes a reduction in oxygen and nutrient delivery to the affected tissues. This disrupts ATP generation via oxidative phosphorylation, causing cells to alter their metabolism and impairs energy-dependent cellular function.

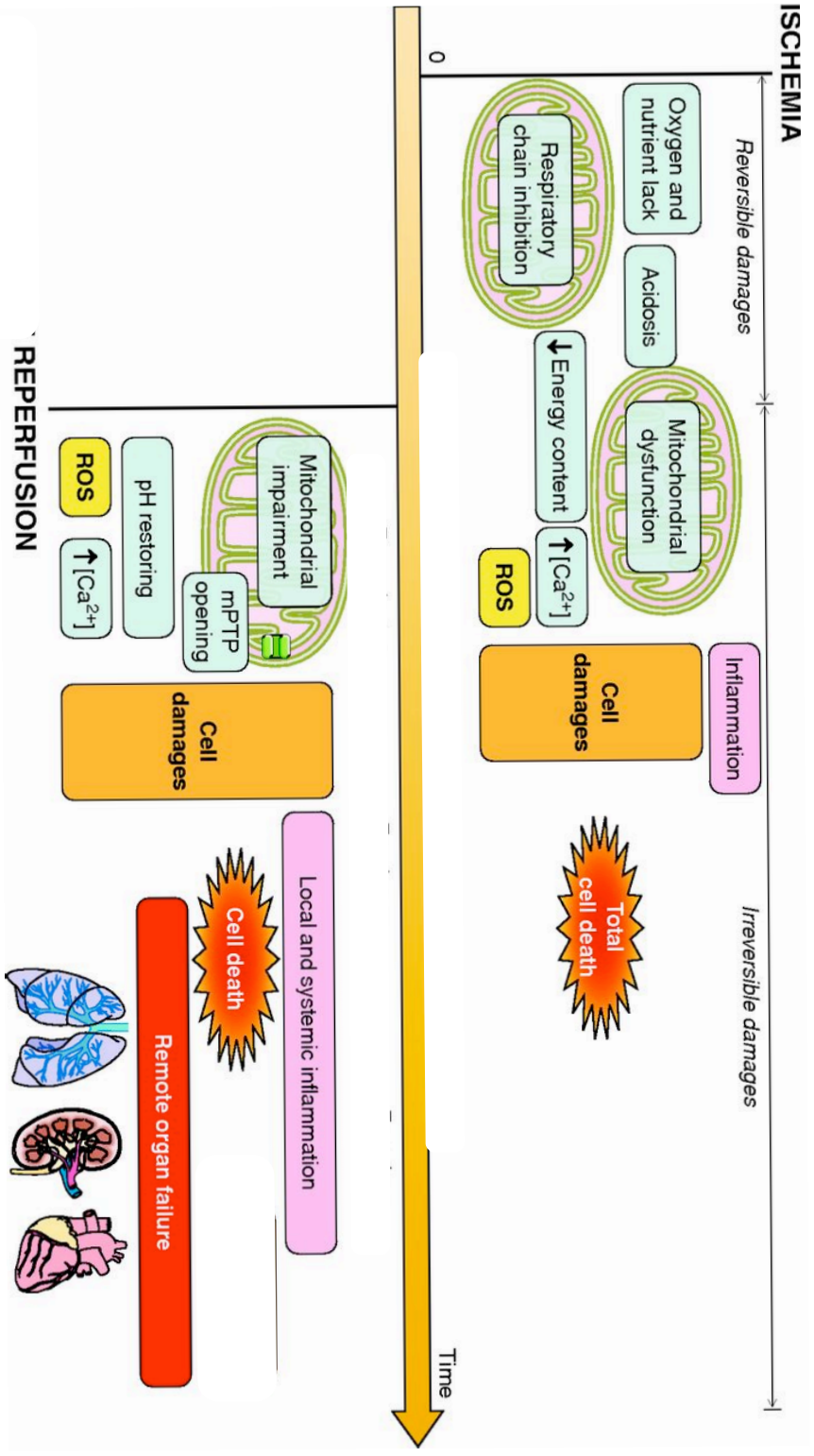

Figure 1: Schematic representation of I/R injury phases.

Reduced ATP availability limits ion pumps in cell membranes, resulting in calcium overload, structural disorganization, and apoptotic, necroptotic, and necrotic cell death. In addition, ischemia induces conformational changes in enzymes such as xanthine oxidase and elicits the formation of pro-inflammatory mediators and expression of adhesion molecules that promote leukocyte/ endothelial cell adhesive interactions (*Eltzschig HK et al 2011, Perrelli MG et al 2011*). These latter processes do not directly contribute to injury during the ischemic phase, but rather set the stage for the second stage of I/R injury (ie, that due to reperfusion), wherein tissue injury is exacerbated when the blood supply is re-established. Paradoxically, the lack of oxygen during ischemia and the replenishment of oxygen during reperfusion both contribute to the total injury sustained by tissues subjected to I/R. The clinical outcome is also determined by a third phase of ROS production that occurs during post-reperfusion repair that is characterized by tissue remodeling and adaptation (*Raedschelders K et al 2012*).

Reperfusion injury

The double-edged sword effects of I/R-induced ROS generation may be related to species of ROS produced, the amount of oxidants generated, and the subcellular location and cellular source of their production under a given set of conditions, as well as at what time during the three phases of responses to I/R they are formed (*Kalogeris T et al 2014*).

Reperfusion represents the second phase of I/R injury and precipitates the generation of ROS that is fueled by the reintroduction of molecular oxygen to the tissues. Xanthine oxidase and phagocyte NADPH oxidase-derived oxidants can damage virtually every biomolecule found in cells and tissues (*Robert AM et al 2014*).

ROS induce tissue dysfunction by directly damaging cells via a number of mechanisms including peroxidation of cell membrane and organelle lipids, oxidizing DNA, activation of matrix metalloproteinases and calpains, producing osmotic cell lysis, induction of no-reflow, and causing opening of the mitochondrial permeability transition pore (*Zinkevich NS et al 2011, Kharbanda RK et al 2010, Seidlmayer LK et al 2015*).

ROS may also induce cell dysfunction and death by indirect mechanisms by interacting with NO, fatty acids or free iron to form peroxynitrite, peroxyl radicals, and hydroxyl radicals, respectively, each of which are capable of producing even more cellular damage than superoxide or hydrogen peroxide (Fig.2).

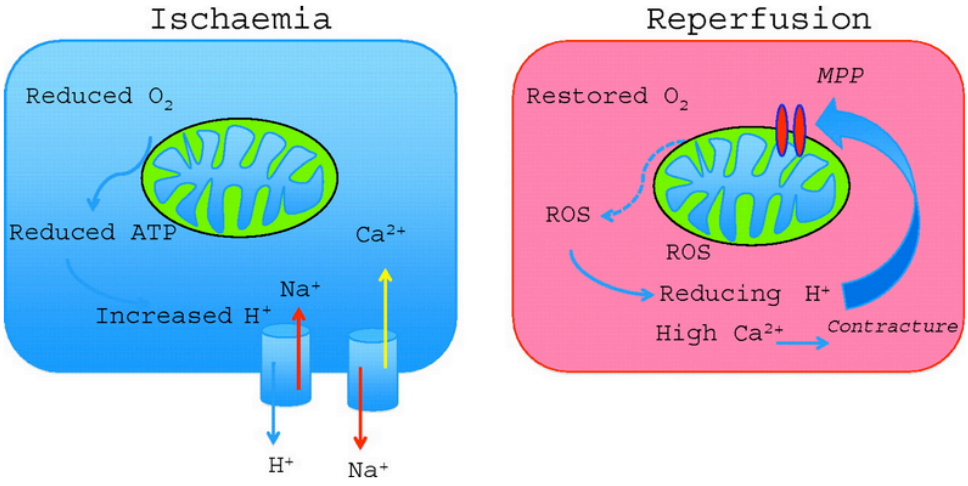

Figure 2: Illustrative scheme of the main mediators of lethal reperfusion injury. As shown in the right, restoring oxygen to ischemic myocardium causes ROS production.

Oxygen paradox

Oxygen-derived ROS also act to enhance the inflammatory response to reperfusion via formation of oxidant-dependent proinflammatory mediators and upregulation of cytokine/chemokine and adhesion molecule expression (*Naha PC et al 2010, Harris J 2010, Naik E et al 2011*) .

Thus, while there is cellular demand for replenishment of oxygen which is met by re-establishing the blood supply, the reintroduction of molecular oxygen to the tissues results in ROS formation that is detrimental to the reperfused tissues. The divergent roles of oxygen in the first two phases of I/R injury are referred to as the oxygen paradox (*Buia LM et al 2010*).

A third ROS paradox arises in later phases of reperfusion, where ROS generation affects several tightly regulated processes that promote organ repair and survival. This third phase constitutes the reparative phase of I/R injury and involves ROS-dependent generation of growth factors that promote angiogenesis, induce proliferation and differentiation of vascular smooth muscle cells to effect vascular remodeling, and promote the activation of matrix metallo-proteinases and other factors that contribute to fibrosis, tissue

remodeling and formation of scar tissue (*Buia et al 2010, Kawaguchi M et al 2011*).

The mitochondria also produce ROS from respiratory chain components, as well as by activation of mitochondrially localized monoamine oxidase, the growth factor adaptor Shc (p66Shc), cytochrome b5 reductase, dihydroorotate dehydrogenase, mitochondrial ATP-sensitive potassium (mKATP) and large-conductance, calcium activated potassium (BKCa) channels, and the Nox isoform designated Nox4. Superoxide normally produced in mitochondria is scavenged by manganese-superoxide dismutase (Mn-SOD or SOD-2) localized in the matrix. In addition, copper/zinc-SOD (Cu/ZN-SOD or SOD-1), which is typically considered a cytoplasmic isoform, is also located in mitochondria between its inner and outer membranes (*Arany I et al 2011, Finkel T et al 2011*, *Daiber et al 2010*).

These SODs dismutase superoxide to less reactive hydrogen peroxide, which can be further metabolized to water and oxygen by the catalytic activity of catalase and glutathione peroxidase.

Mitochondrial uncoupling proteins also serve to reduce the production of ROS by causing mitochondrial depolarization, which reduces the potential driving electron transfer and by allowing

protons to reenter the matrix, thereby bypassing ATP synthase (*Mailloux RJ et al 2011*).

Short-term opening is involved in cardioprotection that involves transient ROS formation (*Perrelli MG et al 2011*).

In contrast, long-lasting mPTP opening, which is facilitated by restoration of pH, calcium overload and the burst of ROS formation at the onset of reperfusion, is followed by profound and irreversible alterations in cellular bioenergetics. Sustained pore formation results in increased mitochondrial permeability to ions and other solutes up to molecular weights of 1.5 kD and collapse of the mitochondrial membrane potential. This is rapidly followed by ATP and NAD^+ depletion, release of accumulated mitochondrial calcium, matrix swelling and outer mitochondrial membrane rupture, which in turn results in loss of pyridine nucleotides, release of pro-apoptotic factors, and further inhibits electron flow through the electron transport chain.

The massive release of ROS during reperfusion requires the involvement of the mPTP in a ROS-induced ROS release positive feedback loop (*Zorov DB et al., 2014*). It is widely believed that mPTP is thus a major causative event in reperfusion injury and cell death.

This concept is consistent with the observation that cardioprotective interventions all seem to intersect at inhibition of the mPTP as an end-effector of enhanced tolerance to I/R.

Because O^{2-} production could be mediated by complex IV during ischemia, the goal of this study is to asses if the administration of ATTM, a new class of sulfide-releasing drugs, may confer protection by inhibiting the complex IV of the respiratory chain, reducing both ROS production and membrane potential depolarization.

Biological role of hydrogen sulfide (H_2S)

H2S is a colourless, flammable gas with a characteristic odour of rotten eggs. It is soluble in water (1 g in 242 ml at 20°C). In water or plasma, H2S is a weak acid which dissociates as follows: H2S ↔ HS− + H+. The pKa at 37°C is 6.76; therefore, when either sodium hydrosulfide (NaHS) or H2S is dissolved in physiological solution (pH 7.4, 37°C), it will form approximately 18.5% H2S and 81.5% hydrosulfide anion (HS), as predicted by the Henderson–Hasselbach equation. H2S is a highly lipophilic molecule and freely penetrates cells of all types. It is this property which endows H2S with, at least the potential for, biological activity.

H2S is formed in mammalian cells largely by the activity of two pyridoxal phosphate-dependent enzymes, cystathionine γ lyase (CSE, EC 4.4.1.1) and cystathionine β synthetase (CBS, EC 4.2.1.22) (Fig. 3). These enzymes are widespread in mammalian tissues and cells and also in many invertebrates and bacteria.

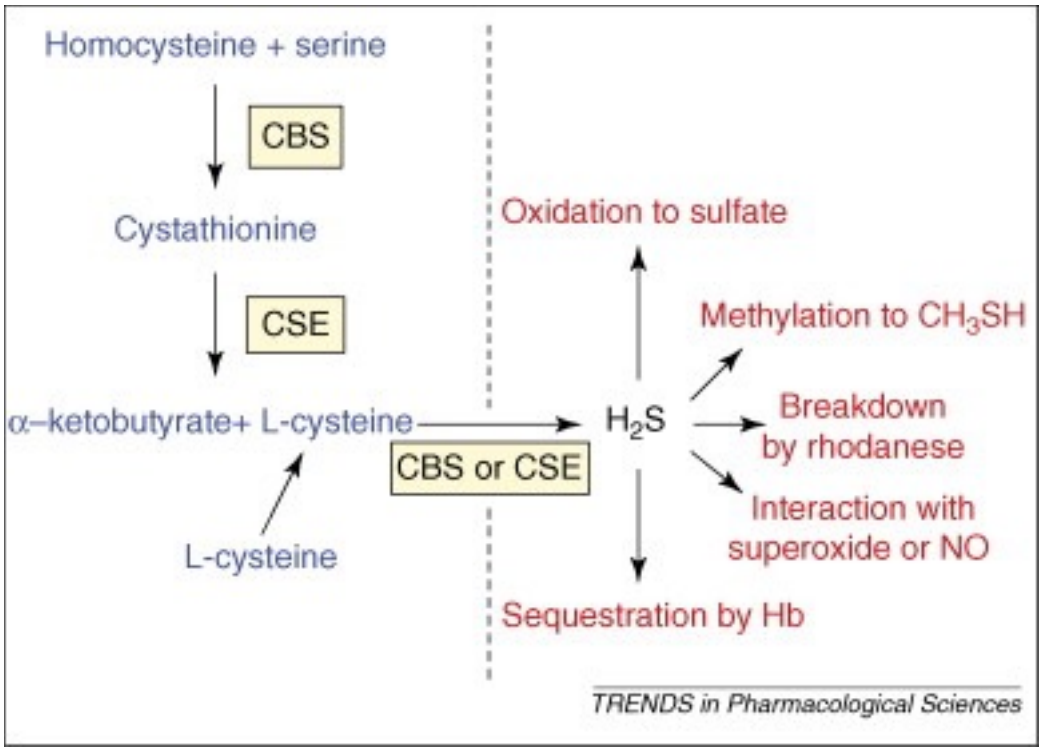

Figure 3: Scheme of representative pathways for H_2S biosynthesis (blue) and catabolism (red) in mammalian tissues.

The pathway(s) by which H_2S is broken down in the body is/are partially known, although several alternatives have been identified. H_2S is rapidly oxidized in mitochondria to thiosulfate, which is then

further converted to sulfite and sulfate (the major product). Whether this is how H_2S is biologically deactivated is not known. However, the majority of sulfate in urine is believe to derive from the direct oxidation of cysteine by cysteine dioxygenase activity and, as such, sulfate cannot be used as a marker for the presence of H_2S in the same way, for example, that nitrate (and nitrite) are often used as markers of NO.

H_2S also undergoes methylation in the cell cytosol by thiol S-methyltransferase to yield methanethiol and dimethylsulfide, and can bind to methaemoglobin to form sulfhaemoglobin.

H_2S interacts with membrane and cytosolic proteins to produce reactive and unstable persulfides. These persulfides can be further converted to other biochemical forms including thiocysteine, thiotaurine, protein–SSH, thiocystine, mercaptopyruvate and others. Sulfide donors may impart structural changes in proteins through persulfide related sulfuration and sulfhydration reactions of proteins.

It is interesting to note that the physiological functions of H2S are comparable to that of NO, as well as controversy surrounding their cytoprotective roles. Throughout the literature on H2S it is reported that the cytoprotective and antioxidant effects occur in the

micromolar range; whereas higher H_2S exposures, i.e., in the millimolar range, potentiate redox stress and are cytotoxic. In the coming future, it is most likely that the field will come to realize the cellular and signalling function and physiological potency of low nanomolar concentrations of H_2S, and that various biochemical forms of the molecule serve important roles in regulating H_2S bioavailability and cellular redox balance. From the information above, it is safe to say that H_2S serves as a proverbial “double-edged sword,” where it can be extremely beneficial or harmful depending on its concentration and cellular location. These observations also reveal how crucial it is moving forward to accurately determine and control for the levels of H_2S in experimental settings, reinforcing the need for rigorous and reliable measurement techniques to monitor the biological levels of H_2S. Finally, increased clarity regarding sulfide cellular signaling will also alleviate confusion and lead to a better understanding of the effects of H_2S administration in biological systems. The future of H_2S biochemistry, chemical biology and pathophysiology represent fertile territory in which to better understand redox processes that will ultimately be important for human health and disease.

Ammonium tetrathiomolybdate (ATTM) mechanism of action

Ammonium tetrathiomolybdate (ATTM) was found to be a slow H_2S releasing agent, in a characteristic time, temperature and pH dependent manner. First synthesis report was almost two centuries ago (*Berzelius 1826*) and since then has been used in men to treat Wilson's, autoimmune, fibrotic and cancer diseases, but has been also used in animals as a copper chelator. However the real efficacy seems related to its anti-inflammatory and anti-angiogenic effects.

Hydrogen sulfide (H_2S) represents the most recently identified endogenously produced gaseous messenger. Although long considered a noxious gas with wide-ranging cytotoxic effects, there is now an accumulation of scientific evidence that H_2S plays a prominent role in cellular signaling. In recent years, the cytoprotective effects of endogenous and exogenous H_2S have been investigated in models of *in vitro* and *in vivo* ischemic injury. H_2S has also been shown to increase KATP channel currents in isolated smooth muscle cells (Zhao W et al 2001) but the mechanism behind the cardioprotective effects of H2S is not limited to modulation of KATP channels and Ca^{2+} handling.

There is much evidence to suggest that H_2S also has anti-apoptotic roles in the cell during M/IR trough activation of two important cell survival pathways, extracellular signal-regulated kinase (ERK1/2)/ mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3-kinase) (*Hu I et al 2008*).

The life of a cell is also dependent on the degree of mitochondrial functionality. During I/R, mitochondria are subjected to oxygen deprivation, reactive oxygen species (ROS) overproduction, and mitochondrial membrane potential ($\Delta\Psi m$) depolarization. Has been shown that H_2S at high levels (80 ppm) can induce a state of hypothermia in mice by inhibiting cytochrome c oxidase, decreasing their metabolic rate and core body temperature trough the induction of a “suspended animation” state, that can prevent ischemic damage to cells (*Roth MB et al 2005*). During myocardial ischemia, ROS production is accelerated and all of the cell’s antioxidants become depleted. H2S is a cytochrome C oxidase inhibitor and therefore (*Hill BC et al 1984*) inhibiting respiration has been shown to decrease the production of ROS thereby avoiding lost of $\Delta\Psi m$. Thus H_2S, at low concentrations, can decrease the production of ROS and preserve mitochondrial function.

Results

One particular aspect of cardiomyocyte injury during ischemia/ reperfusion is the disruption of mitochondrial function, indeed, the maintenance of oxidative phosphorylation for preventing myocyte death after ischemic injury has long been recognized as a critical event after myocardial injury (MI). In the context of the continuously high energetic demand of the heart, the loss of Mitochondrial membrane potential ($\Delta\psi m$) causes a rapid impairment of mitochondrial and cellular function that can lead to necrotic or apoptotic cell death. Thus, also the maintaining of the $\Delta\psi m$ is of paramount importance.

We conducted 4 experiments in order to evaluate the effects of ATTM on H9C2 cells, comparing by the use of Annexin V/ PI assay the percentage of viable cells with and without the drug after 24hrs anoxia/ 2hrs reoxygenation. The results show that ATTM seems increase cell survival in a dose-dependent manner (Fig. 1) also reducing the percentage of late apoptotic cells.

The postulated protective effect of ATTM on mitochondria is based on its antioxidative effect, deacreasing reactive oxygene species (ROS)

production by blocking CIV of the mitochondrial electron transport chain.

We then measured superoxide levels by MitoSOX Red in three separate experiments, using the same I/R protocol.

Histograms of FACS analysis showed marked increase of in mitochondrial fluorescence intensity of MitoSOX in H9c2 cells treated with Antimycin A (Fig.2). Quantitative measurements of the mean fluorescence intensities from the samples demonstrated fold increase in MitoSOX fluorescence intensity of 2 ± 0.5 with AntA, 0.7 ± 0.17 following 24h hypoxia/2h reoxygenation without drug and a significant reduction with ATTM 5.5mM.

Conclusions

In the current study we show that administration of ATTM at the time of reperfusion limits the extent of myocyte death in our *in vitro* I/R model. The study of the preservation of mitochondrial function in myocardial cytoprotection is of paramount importance. Mitochondria play a central role in the development of reperfusion injury because the recovery of pH, oxidative stress, and calcium overload induce abrupt opening of mitochondria permeability transition pores (mPTPs), high conductance megachannels that are localized to contact sites between the inner and outer mitochondrial membranes (*Baines CP, 2010*). When opened, mPTPs permit communication between the cytoplasm and the mitochondrial matrix. While low pH during ischemia prevents opening of the megachannel, oxidative opening of the mPTP is critical to reperfusion injury. Depending on a complex balance among cellular inducers and antagonists, the open probability of the mPTP can be transient or long-lived.

H_2S known to be a potent and reversible inhibitor of cytochrome *c* oxidase (complex IV of the mitochondrial electron transport chain), in addition to the ability to modulate a whole organism metabolism inducing a suspended animation-like state in mice, conducts also

cardioprotection by inhibiting the mitochondrial respiration during reperfusion.

The inhibition of mitochondrial respiration has been shown (*Chen Q et al 2006*) to protect against myocardial I/R injury by limiting the generation of reactive oxygen species and diminishing the degree of mitochondrial uncoupling leading to decreased infarct size and preserved function.

In conclusion ATTM shows protection against the increased ROS production and the drop of cell viability during Ischaemia/ Reperfusion Injury in our in vitro model. Because the majority of reactive oxygen species derive from mitochondria both during ischaemia and reperfusion phases, leading to cell dysfunction and direct damage of lipids, proteins and DNA, sulfide's ability to modulate metabolism is of particular interest due to the implications this could have for modulation of inflammation and apoptosis, and oxidative stress reduction.

The widespread synthesis of new sulfide's donors with different releasing properties reflects their wide range of applications. ATTM characterized by a slow and consistent release of sulfide, is one the most effective sulfide donors moving towards clinical practice.

Meterial and Methods

Cell culture

Embryonic rat cardiomyoblast, H9C2, have been plated at density of ≈1 x 10^6 in 75 cm^2 flask, cultured at 37°C in 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagles Medium (DMEM 1X, Gibco, 41966-029) and supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma F9665). Cells have been passed regularly and sub-cultured at about 70% for 3 passages before experimental procedure.

Experimental protocol anoxia/reoxygenation

H9C2 cells have been plated 24hrs before the experiment at density of 60 x 10^3 in 12 well plates.

At the start of the experiment the culture medium have been replaced with 2 mL of DMEM (serum free, in order to mimic hypoxic state). Cells have than been placed into an anaerobic chamber saturated with 95% N_2 and 5% CO_2 for 24 hrs.

Simulated ischemia have been followed by a simulated reperfusion period during wich normoxic fresh culture medium have been added

at the anoxic medium, with and without MGC-0109 (0mM; 0.0055mM; 0.055mM; 0.55mM; 5.5mM) in a normoxic incubator for 120 min .

Flow cytometry

Cell viability apoptosis

Viability and apoptosis have been assessed using Annexin V-Propidium Iodide assay. Cells have been harvested, washed with PBS (no Ca/Mg) and resuspended in 100ul of APC-Annexin V (BD biosciences) binding buffer (150mM NaCl, 10mM HEPES pH7.4, 10mM $CaCl_2$) and stained for 15min at RT in the dark. At the end of the staining 200ul of binding buffer have been added in each sample.

All data have been acquired on FACS Calibur using Cell Quest software and analyzed with Flow Jo (version IX).

Superoxide production

For the determination of mitochondrial superoxide, cells have been loaded with MitoSOX™ Red mitochondrial superoxide indicator for live cells (ThermoFisher, M36008) prepared following the manufacturer's protocol.

Breafly cells have been loaded with 1mL of 5microM MitoSOX™ reagent working solution and incubated for 30min at 37°C in the dark according to Mukhopadhyay P. et al., Nature Protocol, 2007 (**Washing Buffer**: *HBSS1X CaCl/MgCl 5mM*; **Staining Buffer**: *HBSS1X CaCl/MgCl 5mM+1% BSA*) . After incubation cells have been washed, harvested and resuspended for the APC-Annexin V (BD bioscience). Control cells have been treated with Antimycin A 1mg/ml and run at 0-40 minutes time.

All data have been acquired on FACS Calibur using Cell Quest and analyzed with Flow Jo (version IX) software.

Statistics

All experiments have been run in triplicate. All date have been expressed as avarage ± standard deviation and analyzed with One-way ANOVA (Tukey post hoc for normal distribution) using SPSS statistics (IBM). Only p values <0.05 have been considered statistical significant.

LIST OF FIGURES

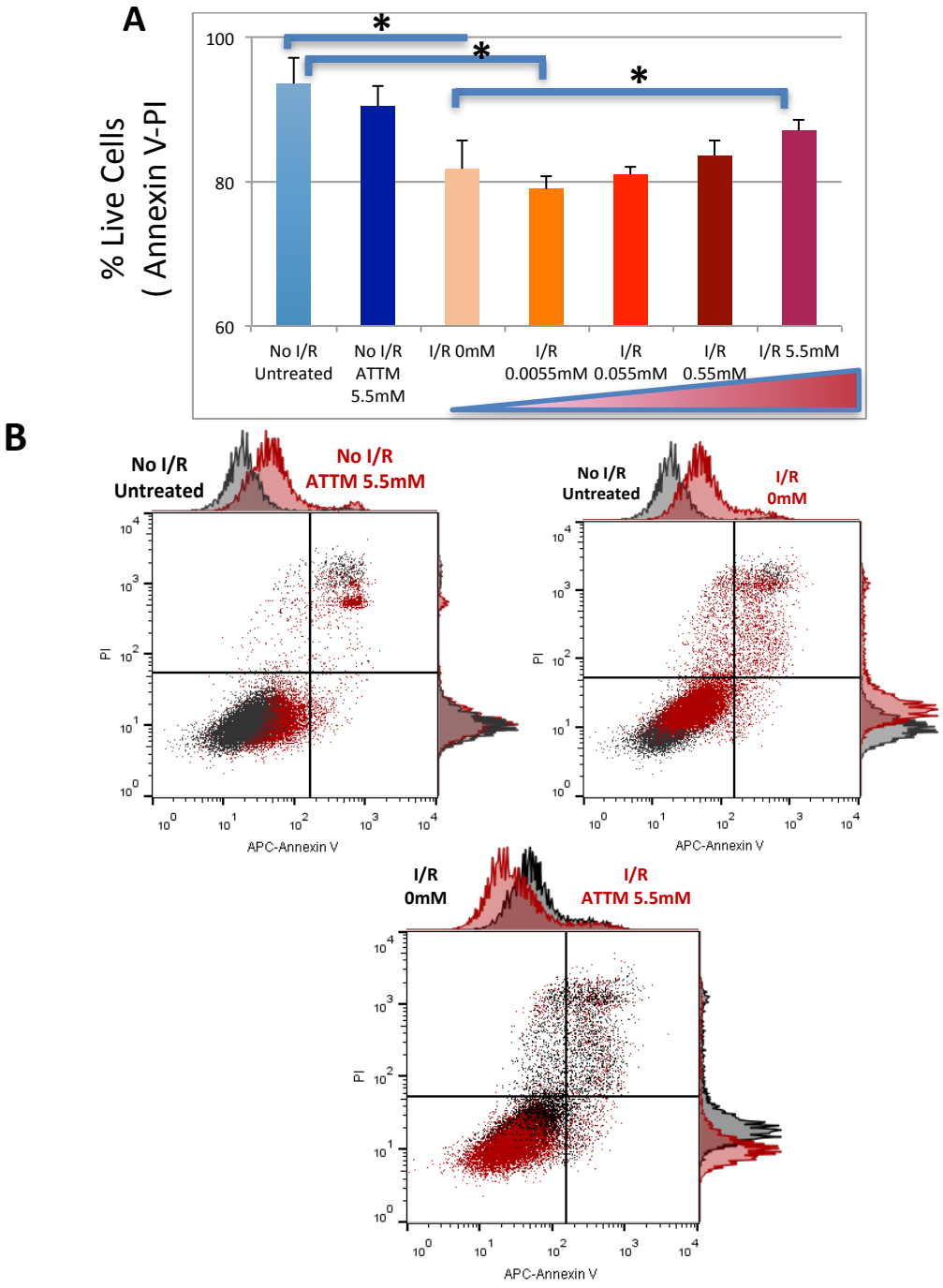

Figure 1: A) Quantitative data expressing percentage of Live Cells (Annexin V negative, PI negative) following simulated I/R injury measured by flow cytometry as indicated. Data presented as average ± SD;*p<0.05. **B)** Determination of cell viability using an Annexin V – PI based assay by flow cytometry.

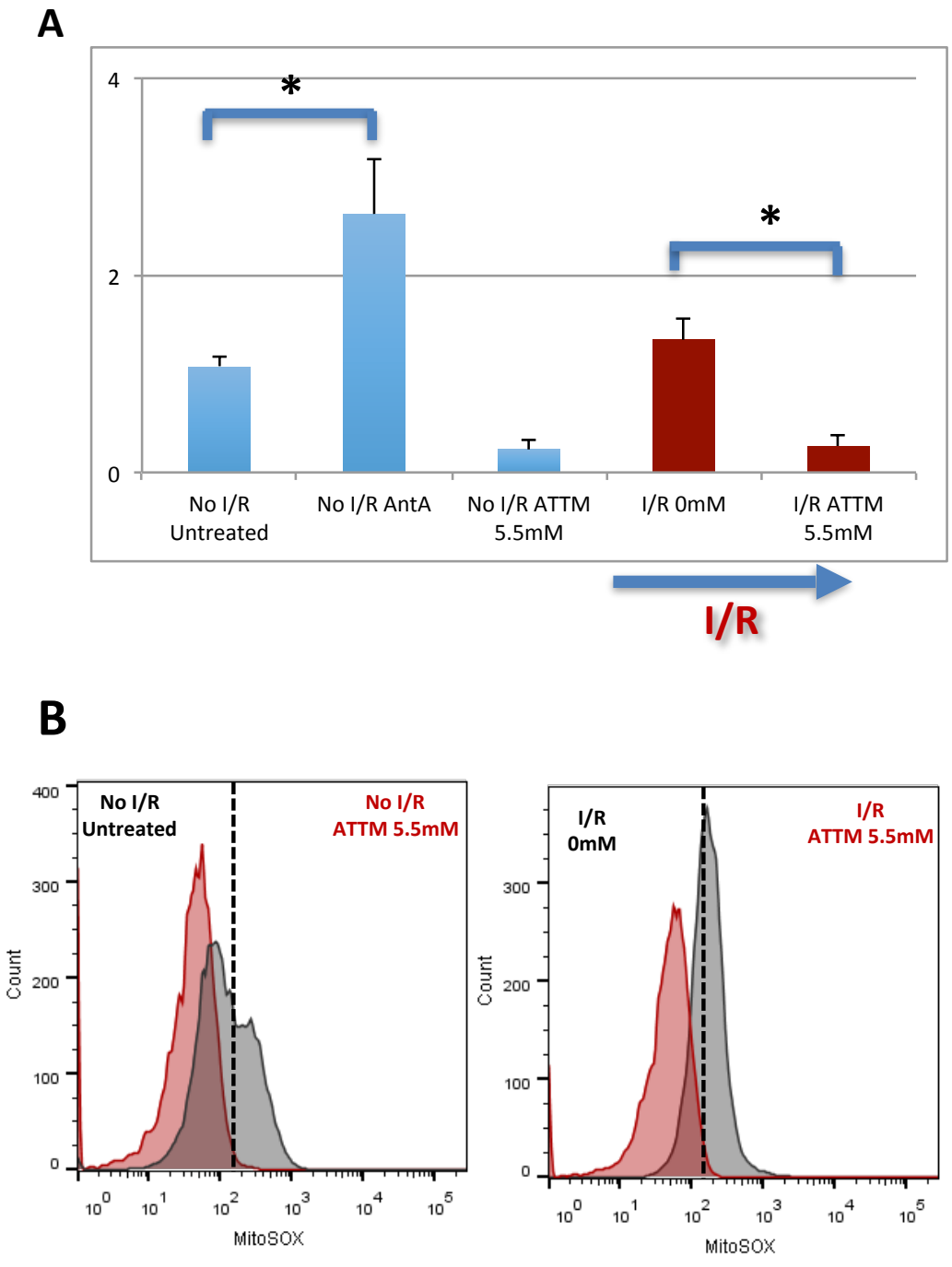

Figura 2: A) Quantitative data expressing fold increase in mean fluorescence intensity of MitoSOX Red following simulated I/R injury measured by flow cytometry as indicated. Data presented as average ± SD; *p<0.05. **B)**Representative histograms of flow cytometry experiments demonstrating increase in mean fluorescence intensity of MitoSOX following simulated I/R injury and treatments as indicated, in H9C2 myocytes.

References

- *Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci. 1996 Feb 1;16(3): 1066-71. PubMed PMID: 8558235.*
- *Aikawa N, Wakabayashi G, Ueda M, Shinozawa Y. Regulation of renal function in thermal injury. J Trauma. 1990 Dec;30(12 Suppl):S174-8. PubMed PMID: 2147724.*
- *Akao M, O'Rourke B, Teshima Y, Seharaseyon J, Marbán E. Mechanistically distinct steps in the mitochondrial death pathway triggered by oxidative stress in cardiac myocytes. Circ Res. 2003 Feb 7;92(2):186-94. PubMed PMID: 12574146.*
- *Ansermino M, Hemsley C. Intensive care management and control of infection. BMJ. 2004 Jul 24;329(7459):220-3. Review. PubMed PMID: 15271835; PubMed Central PMCID: PMC487741.*
- *Arany, I., Faisal, A., Clark, J. S., Vera, T., Baliga, R., & Nagamine, Y. (2010). p66SHC-mediated mitochondrial dysfunction in renal proximal tubule cells during oxidative injury. American Journal of Physiology-Renal Physiology, 298(5), F1214-F1221.*
- *Babior BM. The leukocyte NADPH oxidase. Isr Med Assoc J. 2002 Nov;4(11):1023-4. Review. PubMed PMID: 12489496.*
- *Babior BM. The NADPH oxidase of endothelial cells. IUBMB Life. 2000 Oct-Nov;50(4-5):267-9. Review. PubMed PMID: 11327320.*
- *Bae JY. and Park HH. Crystal structure of NALP3 protein pyrin domain (PYD) and its implications in inflammasome assembly. J Biol Chem 286: 39528–39536, 2011*

- *Baines CP. Role of the Mitochondrion in Programmed Necrosis. Frontiers in Physiology. 2010;1:156. doi:10.3389/fphys. 2010.00156.*
- *Barja, G. (1999) Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J. Bioenerg. Biomembr. 31, 347–366*
- *Barnese K, Gralla EB, Valentine JS, Cabelli DE. Biologically relevant mechanism for catalytic superoxide removal by simple manganese compounds. Proc Natl Acad Sci U S A. 2012 May 1;109(18):6892-7. doi: 10.1073/pnas.1203051109. PubMed PMID: 22505740; PubMed Central PMCID: PMC3344976.*
- *Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996 Nov; 271(5 Pt 1):C1424-37. Review. PubMed PMID: 8944624.*
- *Benko S, Philpott DJ, Girardin SE. The microbial and danger signals that activate Nod-like receptors. Cytokine. 2008 Sep;43(3): 368-73. doi: 10.1016/j.cyto.2008.07.013. Review. PubMed PMID: 18715799.*
- *Boveris A, Oshino N, Chance B. The cellular production of hydrogen peroxide. Biochem J. 1972 Jul;128(3):617-30. PubMed PMID:4404507; PubMed Central PMCID:PMC1173814.*
- *Brusselaers, N., Monstrey, S., Colpaert, K., Decruyenaere, J., Blot, S. I., & Hoste, E. A. (2010). Outcome of acute kidney injury in severe*

burns: a systematic review and meta-analysis. Intensive care medicine, 36(6), 915-925.

- *Buja, L. M., & Weerasinghe, P. (2010). Unresolved issues in myocardial reperfusion injury. Cardiovascular Pathology, 19(1), 29-35.*
- *C. Chinopoulos Mitochondrial consumption of cytosolic ATP: not so fast FEBS Lett., 585 (2011), pp. 1255–1259F*
- *Chance B, Saronio C, Leigh JS Jr. Functional intermediates in the reaction of membrane-bound cytochrome oxidase with oxygen. J Biol Chem. 1975 Dec 25;250(24):9226-37. PubMed PMID: 172505.*
- *Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Reversible blockade of electron transport during ischemia protects mitochondria and decreases myocardial injury following reperfusion. J Pharmacol Exp Ther. 2006 Dec;319(3):1405-12. PubMed PMID: 16990510.*
- *Coon MJ, Ding XX, Pernecky SJ, Vaz AD. Cytochrome P450: progress and predictions. FASEB J. 1992 Jan 6;6(2):669-73. Review. PubMed PMID: 1537454.*
- *Cruz CM., Rinna A., Forman HJ., Ventura AL., Persechini PM., and Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 282: 2871–2879, 2007*
- *Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010 Aug 1;429(3):403-17. doi: 10.1042/BJ20100323. Review. PubMed PMID: 20626350.*

- *D.G. Nicholls, O. Lindberg Inhibited respiration and ATPase activity of rat liver mitochondria under conditions of matrix condensation FEBS Lett., 25 (1972), pp. 61–64*
- *Daiber, A. (2010). Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1797(6), 897-906.*
- *Dal-Pizzol F, Ritter C, Cassol-Jr OJ, Rezin GT, Petronilho F, Zugno AI, Quevedo J, Streck EL. Oxidative mechanisms of brain dysfunction during sepsis.Neurochem Res. 2010 Jan;35(1):1-12. doi: 10.1007/s11064-009-0043-4. Review.PubMed PMID: 19680806*
- *Dostert C., Petrilli V., Van Bruggen R., Steele C., Mossman BT., and Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320: 674–677, 2008*
- *Duewell P., Kono H., Rayner KJ., Sirois CM., Vladimer G., Bauernfeind FG., Abela GS., Franchi L., Nunez G., Schnurr M., Espevik T., Lien E., Fitzgerald KA., Rock KL., Moore KJ., Wright SD., Hornung V., and Latz E. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464: 1357–1361, 2010.*
- *E.I. Rugarli, T. Langer Mitochondrial quality control: a matter of life and death for neurons EMBO J., 31 (2012), pp. 1336–1349*

- *Eltzschig, H. K., & Eckle, T. (2011). Ischemia and reperfusion [mdash] from mechanism to translation. Nature medicine, 17(11), 1391-1401.*
- *Finkel, T. (2011). Signal transduction by reactive oxygen species. The Journal of cell biology, 194(1), 7-15.*
- *Finnerty CC, Herndon DN, Chinkes DL, Jeschke MG. Serum cytokine differences in severely burned children with and without sepsis. Shock. 2007;27:4–9.*
- *Finnerty CC, Herndon DN, Przkora R, Pereira CT, Oliveira HM, Queiroz DM, Rocha AM, Jeschke MG. Cytokine expression profile over time in severely burned pediatric patients. Shock. 2006;26:13–19*
- *Fischer, A. Hamann, H.D. Osiewacz Mitochondrial quality control: an integrated network of pathways Trends Biochem. Sci., 37 (2012), pp. 284–292*
- *Galley, H. F. (2011). Oxidative stress and mitochondrial dysfunction in sepsis. British journal of anaesthesia, 107(1), 57-64.*
- *Gottlieb RA (2000) Mitochondria: execution central. FEBS Lett. 482: 6–12*
- *Gottlieb RA. Role of mitochondria in apoptosis. Crit Rev Eukaryot Gene Expr. 2000;10(3-4):231-9. PubMed PMID: 11272466.*
- *Haigis MC, Yankner BA. The aging stress response. Mol Cell. 2010 Oct 22;40(2):333-44. doi: 10.1016/j.molcel.2010.10.002. Review. PubMed PMID: 20965426; PubMed Central PMCID: PMC2987618.*

- *Harris, J. (2011). Autophagy and cytokines. Cytokine, 56(2), 140-144.*
- *Hausenloy, D. J., & Yellon, D. M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. The Journal of clinical investigation, 123(1), 92-100.*
- *Hill BC, Woon TC, Nicholls P, Peterson J, Greenwood C, Thomson AJ. Interactions of sulphide other ligands with cytochrome c oxidase An electron-paramagnetic-resonance study. Biochem J. 1984;224:591–600.*
- *Hiroyuki Tsutsui, Shintaro Kinugawa, Shouji Matsushima American Journal of Physiology - Heart and Circulatory Physiology Dec 2011, 301 (6) H2181-H2190; DOI: 10.1152/ajpheart. 00554.2011*
- *Hoover L, Bochicchio GV, Napolitano LM, Joshi M, Bochicchio K, et al. (2006) Systemic inflammatory response syndrome and nosocomial infection in trauma. J Trauma 61: 310–316; discussion 316–317.*
- *Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK, Bian JS. Cardioprotection induced by hydrogen sulfide preconditioning involves activation of erk and pi3k/akt pathways. Pflugers Arch. 2008;455:607–616.*
- *Ichijo H, Nishida E, Irie K et al. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science **275**: 90–94*

- *Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science. 1997 Jan 3;275(5296): 90-4. PubMed PMID: 8974401.*
- *Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, Nakada K, Honma Y, Hayashi J. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science. 2008 May 2;320(5876):661-4. doi: 10.1126/science. 1156906. PubMed PMID: 18388260.*
- *J. Nunnari, A. Suomalainen Mitochondria: in sickness and in health Cell, 148 (2012), pp. 1145–1159*
- *Jeremy W. Chambers, Philip V. LoGrasso. Mitochondrial c-Jun N-terminal Kinase (JNK) Signaling Initiates Physiological Changes Resulting in Amplification of Reactive Oxygen Species Generation J Biol Chem. 2011 May 6; 286(18): 16052–16062. Published online 2011 Mar 16. doi: 10.1074/jbc.M111.223602PMCID: PMC3091214*
- *Jinhua Wang, Isere Kuiatse, Adrian V. Lee, Jingxuan Pan, Armando Giuliano, Xiaojiang Cui. Sustained JNK Activity Promotes Epithelial-Mesenchymal Transition, Invasion, and Survival of Breast Cancer Cells by Regulating ERK Activation Mol Cancer Res. Author manuscript; available in PMC 2011 Feb 9.Published in final edited form as: Mol Cancer Res. 2010 Feb; 8(2): 266–277. Published online 2010 Feb 9. doi: 10.1158/1541-7786.MCR-09-0221 PMCID: PMC2824784*

- *Kalogeris, T., Bao, Y., & Korthuis, R. J. (2014). Mitochondrial reactive oxygen species: a double edged sword in ischemia/ reperfusion vs preconditioning. Redox biology, 2, 702-714.*
- *Kawaguchi, M., Takahashi, M., Hata, T., Kashima, Y., Usui, F., Morimoto, H., ... & Hongo, M. (2011). Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. Circulation, 123(6), 594-604.*
- *Koopman WJ, Willems PH, Smeitink JA. Monogenic mitochondrial disorders. N Engl J Med. 2012 Mar 22;366(12): 1132-41. doi: 10.1056/NEJMra1012478. Review. PubMed PMID: 22435372.*
- *Li D, Li X, Wu J, et al. Involvement of the JNK/FOXO3a/Bim Pathway in Neuronal Apoptosis after Hypoxic–Ischemic Brain Damage in Neonatal Rats. Biagini G, ed. PLoS ONE. 2015;10(7):e0132998. doi:10.1371/journal.pone.0132998.*
- *Liochev SI, Fridovich I. Superoxide and iron: partners in crime. IUBMB Life 1999 Aug;48(2):157-61. Review. PubMed PMID: 10794591.*
- *Loschen G, Azzi A. On the formation of hydrogen peroxide and oxygen radicals in heart mitochondria. Recent Adv Stud Cardiac Struct Metab. 1975;7:3-12. PubMed PMID: 179119.*
- *Mailloux, R. J., & Harper, M. E. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free Radical Biology and Medicine, 51(6), 1106-1115.*

- *Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H, Nagai S, Koyasu S, Matsumoto K, Takeda K, Ichijo H. ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nat Immunol. 2005 Jun;6(6):587-92. PubMed PMID: 15864310.*
- *Moens, A. L., Claeys, M. J., Timmermans, J. P., & Vrints, C. J. (2005). Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. International journal of cardiology, 100(2), 179-190.*
- *Naha, P. C., Davoren, M., Lyng, F. M., & Byrne, H. J. (2010). Reactive oxygen species (ROS) induced cytokine production and cytotoxicity of PAMAM dendrimers in J774A. 1 cells. Toxicology and applied pharmacology, 246(1), 91-99.*
- *Naik, E., & Dixit, V. M. (2011). Mitochondrial reactive oxygen species drive proinflammatory cytokine production. The Journal of experimental medicine, 208(3), 417-420.*
- *Neuss M, Monticone R, Lundberg MS, Chesley AT, Fleck E, Crow MT. The apoptotic regulatory protein ARC (apoptosis repressor with caspase recruitment domain) prevents oxidant stress-mediated cell death by preserving mitochondrial function. J Biol Chem. 2001 Sep 7;276(36):33915-22. PubMed PMID: 11438535.*
- *Overley-Adamson B., Artlett CM., Stephens C., Sassi-Gaha S., Weis RD., and Thacker JD. Targeting the unfolded protein response, XBP1, and the NLRP3 inflammasome in fibrosis and cancer. Cancer Biol Ther 15, 420–462, 2014*

- *Palmieri Diseases caused by defects of mitochondrial carriers: a review Biochim. Biophys. Acta, 1777 (2008), pp. 564–578F.*
- *Paravicini TM, Touyz RM. Redox signaling in hypertension. Cardiovasc Res. 2006 Jul 15;71(2):247-58. Review. PubMed PMID: 16765337.*
- *Perrelli, M. G., Pagliaro, P., & Penna, C. (2011). Ischemia/ reperfusion injury and cardioprotective mechanisms: role of mitochondria and reactive oxygen species. World J Cardiol, 3(6), 186-200.*
- *Pruitt BA Jr. Protection from excessive resuscitation: "pushing the pendulum back". J Trauma. 2000 Sep;49(3):567-8. PubMed PMID: 11003341.*
- *Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med. 2002 Dec 1;33(11):1451-64.Review. PubMed PMID: 12446202.*
- *Raedschelders, K., Ansley, D. M., & Chen, D. D. (2012). The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacology & therapeutics, 133(2), 230-255.*
- *Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene. 2007 May 14;26(22):3100-12. Review. PubMed PMID: 17496909.*
- *Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol.*

2008;40(12):2707-19. doi: 10.1016/j.biocel.2008.04.009. Review. PubMed PMID: 18562239.

- *Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta. 2016 Dec;1863(12):2977-2992. doi: 10.1016/j.bbamcr. 2016.09.012. Review. PubMed PMID:27646922.*
- *Roth MB, Nystul T. Buying time in suspended animation. Sci Am. 2005;292:48–55.*
- *Sauaia A, Moore FA, Moore EE, Lezotte DC (1996) Early risk factors for postinjury multiple organ failure. World J Surg 20: 392–400.*
- *Shukla V, Mishra SK, Pant HC. Oxidative stress in neurodegeneration. Adv Pharmacol Sci. 2011;2011:572634. doi: 10.1155/2011/572634. PubMed PMID: 21941533; PubMed Central PMCID: PMC3177364.*
- *Sorbara MT, Girardin SE. Mitochondrial ROS fuel the inflammasome. Cell Research. 2011;21(4):558-560. doi:10.1038/cr. 2011.20.*
- *Stanojcic M, Chen P, Harrison RA, et al. Leukocyte Infiltration and Activation of the NLRP3 Inflammasome in White Adipose Tissue Following Thermal Injury. Critical care medicine. 2014;42(6): 1357-1364. doi:10.1097/CCM.0000000000000209.*
- *Takeda K, Shimozono R, Noguchi T, Umeda T, Morimoto Y, Naguro I, Tobiume K, Saitoh M, Matsuzawa A, Ichijo H. Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1. J*

Biol Chem. 2007 Mar 9;282(10):7522-31. PubMed PMID: 17210579.

- *Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T, Ichijo H. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep. 2001 Mar;2(3):222-8. PubMed PMID: 11266364; PubMed Central PMCID: PMC1083842.*
- *Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009 Jul;8(7):579-91. doi: 10.1038/nrd2803. Review. PubMed PMID: 19478820.*
- *Tschopp J. and Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nat Rev Immunol 10: 210–215, 2010*
- *Van Haren, R. M., Thorson, C. M., Valle, E. J., Busko, A. M., Guarch, G. A., Andrews, D. M., ... & Proctor, K. G. (2013). Hypercoagulability after burn injury. Journal of Trauma and Acute Care Surgery, 75(1), 37-43.*
- *Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH and Thompson CB (2001) Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol. Cell. Biol. 21: 5899–5912*
- *Vignais PV. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol Life Sci. 2002 Sep;59(9):1428-59. Review. PubMed PMID: 12440767.*

- *Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev. 15: 2922–2933*
- *Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007 Apr;19(2):142-9. Review. PubMed PMID: 17303404.*
- *Wiggins-Dohlvik K, Han MS, Stagg HW, Alluri H, Shaji CA, Oakley RP, Davis ML, Tharakan B. Melatonin inhibits thermal injury-induced hyperpermeability in microvascular endothelial cells. J Trauma Acute Care Surg. 2014 Dec;77(6):899-905; discussion 905. doi: 10.1097/TA.0000000000000346. PubMed PMID: 25051382.*
- *Win S, Than TA, Fernandez-Checa JC, Kaplowitz N. JNK interaction with Sab mediates ER stress induced inhibition of mitochondrial respiration and cell death. Cell Death & Disease. 2014;5(1):e989-. doi:10.1038/cddis.2013.522.*
- *Yang Y, Jiang S, Dong Y, Fan C, Zhao L, Yang X, Li J, Di S, Yue L, Liang G, Reiter RJ, Qu Y. Melatonin prevents cell death and mitochondrial dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. J Pineal Res. 2015 Jan;58(1):61-70. doi: 10.1111/jpi.12193. PubMed PMID: 25401748.*
- *Yaser Azizi, Mahdieh Faghihi, Alireza Imani, Mehrdad Roghani, Afshin Nazari, Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction, Peptides, Volume 46, August 2013, Pages 76-82, doi.2013.05.006.*

- *Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of h(2)s as a novel endogenous gaseous k(atp) channel opener. EMBO J. 2001;20:6008–6016.*
- *Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature, 469(7329), 221-225.*
- *Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiological Reviews. 2014;94(3):909-950. doi:10.1152/physrev.00026.2013.*