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CICLO XXIX

CHARACTERIZATION OF THE ACTIVATION OF MONOAMINE OXIDASES IN CONDITIONS OF CARDIAC DAMAGE AND INFLAMMATION

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2. ABBREVIATIONS

ATP adenosine triphosphate
CNS central nervous system
DCFDA 2′,7′-Dichlorofluorescin diacetate
DHE dihydroethidium
ERK extracellular signal-regulated kinase
ETC electron transport chain
GM-CSF growth macrophage colony stimulating factor
HA histamine
H_2O_2 hydrogen peroxide
IFNγ interferon γ
IL- interleukin
IMM inner mitochondrial membrane
IR ischemia reperfusion
JNK c-Jun N-terminal kinase
LDH lactate dehydrogenase
LPS lipopolysaccharide
MAO monoamine oxidase
MAPK mitogen activated protein kinases
M-CSF macrophage colony stimulating factor
Mn monocytes
MS mass spectrometry
MTR mitotracker red
MΦ macrophages
NE norepinephrine
NMH N^{τ}-methylhistamine
NOX nicotinamide adenine dinucleotide phosphate oxidase
NRVMs neonatal rat ventricular myocytes
OMM outer mitochondrial membrane
PEA phenylethylamine
Pi phosphate
PNS peripheral nervous system
PTP permeability transition pore
ROS reactive oxygen species
SDS sodium dodecyl sulphate
TNF-α tumor necrosis factor α
5-HT serotonin
15-LOX1 15-lipoxygenase

3. SUMMARY

Mitochondria represent the main site of both ROS formation and degradation. One of the main source of mitochondrial ROS is represented by monoamine oxidases (MAOs). MAOs are flavoenzymes located in the outer mitochondrial membrane, which catalyse the oxidative deamination of biogenic amines yielding aldehydes, ammonia, and hydrogen peroxide (H_2O_2). It has been demonstrated that these products contribute to the oxidative stress occurring in hearts subjected to pathological conditions, such as cardiac reperfusion injury and decompensated hypertrophy. As MAO inhibitors significantly protect the heart in these models of cardiac injury, we focused our attention on the molecular mechanisms underlying MAO activation. To address this issue we investigated (i) the availability of MAO substrates under conditions of oxidative stress or injury and (ii) their main cellular sources in the whole heart. By mass spectrometry (MS) analysis we identified several amines that became available upon different *ex vivo* protocols of cardiac injury, and we focused our attention on N^{τ}-methylhistamine (NMH) and its precursor histamine, which were found the most abundant. Thus, we evaluated the contribution of myocyte and non-myocyte cells as possible sources of MAO substrates. For this purpose we firstly excluded synaptic terminals that innervate heart by mice injection of a neurotoxin, 6-hydroxydopamine (6-OH-DOPA). Our findings demonstrate the relevant contribution of synaptic terminals to increase the availability of MAO substrates in the *ex vivo* model of oxidative stress. Next we considered isolated cardiomyocytes, as these cells undergo MAO-dependent oxidative stress. We showed that cardiomyocytes can synthesize MAO substrates and to induce MAO activity when exposed to oxidative stress. Taken together, we establish for the first time a relevant interaction between histamine metabolism and MAO activity in cardiac injury, that does not involve a receptor-dependent pathway. These findings can explain how MAO activity is turned on when cells are stressed. Moreover, it also helps understanding how the increase in MAO activity amplifies an initial oxidative stress.

It has been largely shown that inflammation is usually involved in cardiac diseases and monocytes (Mn), macrophages (MΦ) as well as mast cells are involved in the inflammatory response. We focused our attention on immune phagocytic cells since the role of MAO has not been conclusively defined. Firstly, we demonstrated that both the MAO isoforms were expressed in both M1 and M2 MΦ. We focused our attention on MAO A, as it resulted the most expressed and significant isoform, especially in M2 MΦ. Then, we characterized its pathway of induction considering two different stimuli: LPS, that is a pro-inflammatory signal and IL-4 *plus* IL-13 (IL-4+IL-13), that is a combination of anti-inflammatory cytokines.

It is well known that during differentiation and polarization MΦ generate ROS, which leads to the activation of different signalling pathways. However, the mechanisms that induce ROS formation and activate this signalling remain unclear. Here we demonstrated that MAO contributes to macrophage differentiation and polarization through its H_2O_2 production.

Taken together, these data demonstrate for the first time that MAO-A plays a role in M2 MΦ differentiation and activation, besides its role in amine oxidative degradation. These novel insights about MAO activity in phagocytic cells suggest

that this enzyme could represent a new target to modulate MΦ differentiation and activation under pathological conditions to avoid the side-effects related to inflammation such as fibrosis, cardiac remodelling, and oxidative stress amplification in post-ischemic reperfusion injury.

3.1 SOMMARIO

I mitocondri rappresentano il sito principale di origine e smaltimento delle specie reattive dell'ossigeno (ROS). Una delle più importanti fonti mitocondriali di ROS è rappresentata dalle monoammino ossidasi (MAO). Le MAO sono flavoenzimi situati nella membrana mitocondriale esterna, che catalizzano la deaminazione ossidativa di ammine biogeniche, producendo aldeidi, ammoniaca e perossido d'idrogeno (H_2O_2). È stato dimostrato che questi prodotti contribuiscono allo stress ossidativo che si verifica in cuori soggetti a condizioni patologiche, come ad esempio l'ischemia riperfusione e lo scompenso da ipertrofia. Dato che gli inibitori per le MAO proteggono significativamente il cuore in questi modelli di danno cardiaco, abbiamo focalizzato la nostra attenzione sui meccanismi molecolari alla base dell'attivazione di questi enzimi. A questo scopo è stata analizzata (i) la disponibilità dei substrati per le MAO in condizioni di stress ossidativo o danno cardiaco, e sono state valutate (ii) le principali fonti cellulari per questi substrati nel cuore. Mediante spettrometria di massa (MS) sono state identificate diverse ammine, rese disponibili in due differenti protocolli sperimentali d'induzione di stress cardiaco *ex vivo*. Particolare attenzione è stata data alla N^{τ}-Metilistamina (NMH) ed il suo precursore istamina, che rappresentavano la maggiore frazione di ammine sul contenuto totale nel cuore in queste condizioni di stress. Quindi, è stato valutato il contributo delle cellule miocitiche e non, come possibili fonti di substrati per le MAO. Anzitutto sono stati considerati i terminali sinaptici che innervano il cuore, e quindi eliminati iniettando gli animali con una neurotossina, la 6-idrossidopamina (6-OH-DOPA). I risultati così ottenuti dimostrano il contributo dei terminali nell'aumentare la disponibilità di substrati per le MAO nel modello *ex vivo* sottoposto a stress ossidativo. Sucessivamente, abbiamo considerato i cardiomiociti isolati, in quanto anch'essi subiscono lo stress ossidativo dipendente dalle MAO. Infatti abbiamo dimostrato che i cardiomiociti soggetti a stress ossidativo sono in grado di sintetizzare substrati per le MAO, promuovendo l'attività dell'enzima. Si stabilisce così per la prima volta un'importante relazione tra il metabolismo dell'istamina e l'attività dell'enzima MAO in condizioni di danno cardiaco, la quale non comporta alcuna dipendenza da un eventuale recettore istaminergico. Questi risultati spiegano come lo stress cellulare possa indurre l'attività enzimatica dell'enzima MAO amplificando l'iniziale stress ossidativo.

È stato ampiamente dimostrato che l'infiammazione insorge durante le malattie cardiache e non solo, inoltre che le cellule infiammatorie coinvolte come monociti (Mn), macrofagi (MΦ) e mastociti sono particolarmente rilevanti ed attive. Pertanto la nostra attenzione è stata incentrata dalle cellule fagocitarie in cui il ruolo dell'enzima MAO non è stato ancora definito. In primo luogo, è stato dimostrato che entrambe le isoforme MAO (A e B) sono espresse sia nei MΦ M1 che M2. Poi ci siamo focalizzati su MAO A, in quanto è risultata essere l'isoforma principalmente espressa, soprattutto nei MΦ M2. In seguito è stato caratterizzato il suo meccanismo d'induzione, considerando due diversi stimoli: l'LPS e la combinazione delle citochine anti-infiammatorie IL-4 ed IL-13 (IL-4 + IL-13).

È noto che, durante i processi di differenziamento e di polarizzazione, i MΦ generino ROS, che inducono l'attivazione di diverse vie di segnale. Tuttavia, i

meccanismi che inducono la formazione di questi ROS non sono stati caratterizzati.
In questo lavoro è stato dimostrato per la prima volta che l'isoforma A dell'enzima MAO svolge un ruolo rilevante nel differenziamento e nell'attivazione dei MΦ M2, mediante la produzione di H_2O_2. Questi nuovi risultati nelle cellule fagocitiche suggeriscono che questo enzima potrebbe rappresentare un nuovo bersaglio per modulare il differenziamento e l'attivazione dei MΦ. In particolare, in condizioni patologiche la loro modulazione potrebbe limitare gli effetti collaterali legati all'infiammazione, come ad esempio la fibrosi, il rimodellamento cardiaco, e l'amplificazione dello stress ossidativo nel danno da ischemia/riperfusione.

4. INTRODUCTION

4.1 Reactive Oxygen Species (ROS)

Oxygen represents an indispensable molecule for any aerobic organisms but it can become toxic when it is partially reduced.
The complete reduction of O_2 into two molecules of H_2O requires four electrons. This process occurs sequentially, since O_2 accepts only one electron at a time. Consequently, O_2 reduction inevitably implies the formation of partially reduced intermediates. In particular, the addition of the first electron yields superoxide anion ($O_2^{\cdot-}$) that becomes hydrogen peroxide (H_2O_2) when one additional electron is added. A further single electron reduction produces the hydroxyl radical (·OH) that accepting another electron becomes H_2O. $O_2^{\cdot-}$ and ·OH are oxygen radicals (i.e, containing unpaired electrons) and represent quite reactive species. H_2O_2 is less reactive *per se*, but, according to the Fenton reaction, in the presence of Fe^{2+} or Cu^{+} generates ·OH. These partially reduced forms of oxygen are commonly referred to as ROS, which also include the highly reactive singlet oxygen ($^1O_2$). This excited form of oxygen is produced by the Haber–Weiss reaction (i.e, the non-enzymatic dismutation of $O_2^{\cdot-}$) or high energy irradiation (i.e, UV light).
ROS are produced at various intracellular sites, yet it is generally accepted that in cardiac myocytes the largest amount of ROS are formed within mitochondria.[1, 2, 3, 4, 5] Besides impairment of energy metabolism and ionic homeostasis, formation of ROS represents an additional process through which mitochondrial dysfunction accelerates, or even determines, the evolution of cell injury towards necrosis or apoptosis.[6, 7, 8, 9, 10, 11]

4.2 Mitochondria as source and target of ROS

Mitochondria are well known as the powerhouse of the cell, as they are responsible for generating most of the ATP in the cell, for their role of posttranslational modifications in the regulation of metabolism, for the regulation and role of mitochondrial matrix calcium. Mitochondria are also key regulators of cell death. In the process of electron transport to generate ATP, mitochondria can be a major source of reactive oxygen species (ROS) that can both contribute to cell death and serve as a signaling molecule.[4, 12]
The large majority of oxygen delivered to mitochondria is fully reduced to water at the level of Complex IV. In this terminal step of the mitochondrial respiratory chain, oxygen is reduced sequentially, yet reaction intermediates remain bound to Complex IV, so that only the final product, i.e, water, is released. Nevertheless, electrons flowing through the respiratory chain can be donated to oxygen at other sites, but in these cases the reduction is not complete resulting in the release of partially reduced forms, especially superoxide anion. In fact, $O_2^{\cdot-}$ is formed at the level of Complex I and III and is then rapidly dismutated into H_2O_2 by SOD.[13] Besides the classical notion of the mitochondrial localization of MnSOD or SOD-2, also CuZnSOD (SOD-1), commonly referred to as the cytosolic isoform, is present in the intermembrane space of mitochondria.[14]

Mitochondrial ROS formation is favored by a decrease in electron flow resulting from respiratory chain inhibition and is counteracted by uncoupling that is generally produced by an increased IMM permeability to protons. This latter process explains the protective efficacy related to increased expression of uncoupling proteins (UCP), especially UCP2 and 3.[15] The level of these proteins is suggested to remain below that required for reducing the inner mitochondrial membrane potential and ATP synthesis, so that the most relevant effect would remain a decrease in ROS formation. However, despite the large body of evidence supporting the cardioprotective role of UCPs,[15] negative results have also been associated with UCP2 overexpression in cardiomyocytes.[16]

Nitric oxide ($NO^{\cdot}$) is a free radical that might modulate mitochondrial ROS formation by inhibiting cytochrome oxidase.[5, 17, 18, 19] This reversible process can be transformed into irreversible alterations of respiratory chain when $NO^{\cdot}$ formation is sustained. Indeed, $NO^{\cdot}$ reacting with $O_2^{\cdot-}$ generates peroxynitrite, which can produce the irreversible nitration of proteins.[20] Interestingly, a proteomic study showed that one-third of the proteins nitrated during inflammatory challenge are of mitochondrial origin.[21]

It must be pointed out that ROS are also produced within mitochondria at sites other than the inner mitochondrial membrane, such as monoamine oxidase (MAO) and p66Shc (fig. 1). These additional mitochondrial processes produce significant amounts of ROS.[22] For instance, in brain mitochondria the highest rate of H_2O_2 formation from respiratory chain, as observed in the presence of the Complex III inhibitor antimycin A, is 48-fold lower than that originating from MAO activity. Therefore, generation of ROS, especially H_2O_2, far from being just an unfortunate side effect of respiration, is catalyzed by specific enzymes, such as MAO and p66Shc.[23]

P66Shc is a vertebrate splice variant of p52Shc and p46Shc, two cytoplasmic adaptor proteins involved in the propagation of intracellular signals from activated tyrosine kinases to Ras.[24] Studies carried out in a wide range of experimental models demonstrated quite clearly that ROS formation is reduced in cells lacking p66Shc, and that systemic and intracellular markers of oxidative stress are diminished in p66Shc $^{-/-}$ mice.[25, 26, 27, 28] Indeed, ROS formation is directly enhanced by p66Shc that, translocating to mitochondria, catalyzes an alternative redox reaction.[27] In particular, electrochemical experiments demonstrated that the amino terminal portion of p66Shc contains a redox active sequence able to couple the reduction of molecular oxygen to H_2O_2 with the oxidation of cytochrome c.[27]

MAOs are enzymes located in the outer mitochondrial membrane. They deaminate key neurotransmitters such as norepinephrine (NE), epinephrine, serotonin and dopamine, generating H_2O_2 alongside.[29] All these monoamines are endowed with potent modulatory effects on myocardial function. Thus, when the heart is subjected to chronic stress, the abundance of circulating/tissue monoamines can make MAO-derived H_2O_2 production particularly prominent. This is the case of acute cardiac damage due to ischemia/reperfusion injury or, on a more chronic stand, of the transition from compensated hypertrophy to overt ventricular dilation/pump failure.

More recently, the list of mitochondrial sources of ROS has been added with NADPH oxidase 4 (NOX4) (fig. 1), suggesting its contribution in oxidative stress and heart failure.[30] However, evidence has also been provided that NOX4 is

localized to the ER and mediates protection against chronic load-induced stress by enhancing angiogenesis.[11, 31]

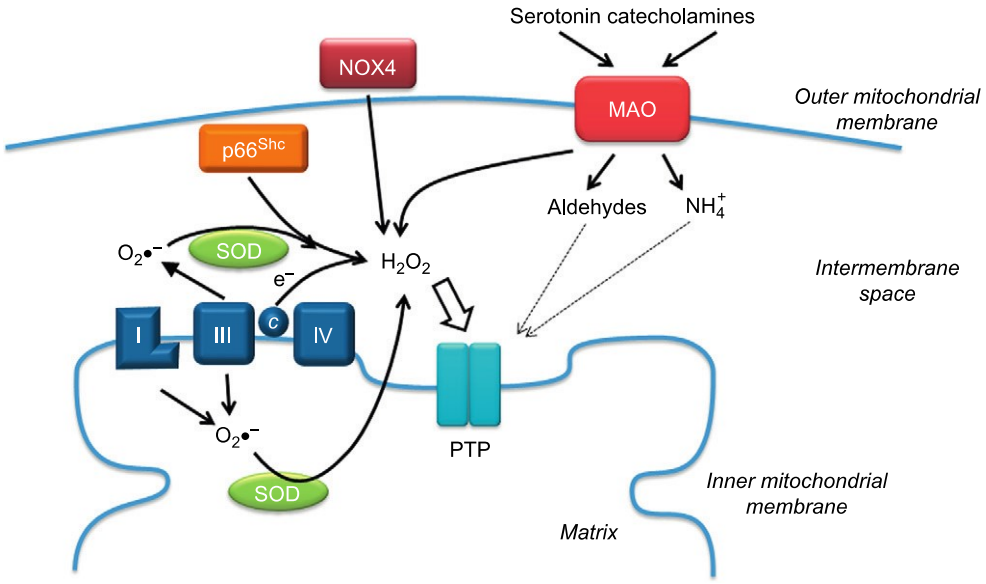

Figure 1. Sources of ROS in mitochondria. *From Di Lisa and Scorrano, Mitochondrial Morphology and Function, Muscle-Fundamental biology and mechanisms of disease, 2012.* [11]

4.3 Mitochondrial dysfunction in cardiac disease

The tight dependence of myocardial metabolism and function on oxygen consumption is clearly indicated by the abundance of mitochondria that occupy more than one-third of the cardiomyocyte volume.

One of the major link between mitochondria and vascular derangements is oxidative stress.[32, 33, 34, 35, 36] In particular, the development of atherosclerosis appears to depend on the mitochondrial metabolism of ROS. In fact, when mitochondrial antioxidant defenses are hampered, atherosclerosis is exacerbated, whereas a decrease in mitochondrial ROS formation reduces atherogenesis.[36] For instance, the absence of SOD-2 increases mtDNA damage and accelerates atherosclerosis in apoE knockout mice.[37] These findings are consistent with results obtained in ischemia/reperfusion experiments, which show that SOD-2 overexpression elicits cardioprotection,[38] whereas a heterozygous deficiency of this enzyme impairs postischemic recovery of the heart.[39]

In addition to the damage to all the cellular components, oxidative stress increases the occurrence of cell death, especially by apoptosis, which greatly contributes to the progress of atherosclerotic lesions. In this respect, an important consequence of ROS accumulation is an increased susceptibility to opening of the mitochondrial permeability transition pore (PTP).[40] PTP opening is especially sensitive to oxidative stress, since it is favored by decreases in $NADPH(H^+)/NADP^+$ and -SH/-S-S ratios.[41, 42] Recent evidence suggests that PTP opening and ROS formation are linked in a vicious cycle. In addition to being a

likely consequence of oxidative stress, PTP opening has been shown to increase mitochondrial ROS formation in cardiac myocytes.[43]
Despite the large body of evidence that relates PTP opening to cell death, especially in the case of an ischemia/reperfusion injury to the heart[44] definitive proof of PTP involvement in atherogenesis is lacking.
While data obtained *in vitro* indicate that PTP opening causes cell death in isolated endothelial and vascular smooth muscle cells[33, 45] no information on the effect of pharmacological or genetic inhibition of the PTP on atherogenesis *in vivo* is available.[46, 47]

4.4 The ischemia/reperfusion injury of the heart

In tissue subjected to ischemia followed by reperfusion (IR), pathologic mechanisms are elicited and produce reversible cell injury and dysfunction, which can progress to irreversible damage if the nature and extent of ischemia is prolonged and/or amplified.[44]
At a cellular level, the onset of ischemia is determined by an insufficient availability of oxygen for mitochondrial oxidations (fig. 2). As a consequence of the reduced or absent oxidative phosphorylation, intracellular creatine phosphate is rapidly depleted with a concomitant rise in Pi, both factors stimulating glycolysis and lactate formation. The accumulation of lactate and the hydrolysis of adenosine triphosphate (ATP) decrease intracellular pH. During the early phase of ischemia, the failure of contraction and the rigor contracture, which are the most relevant changes in myocardial function, are caused by intracellular acidosis and severe reduction in ATP content respectively. Besides these relevant functional changes, several cellular activities are modified by the fall in pH and ATP content, including the inhibition of glycolysis which is observed under conditions of severe ischemia.
A low pH and the accumulation of lactate decrease the activity of both phosphofructokinase (PFK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition, the fall in ATP is associated with membrane binding and inactivation of PFK. Thus, the failure in mitochondrial ATP production, which initially stimulates glycogen breakdown and glycolysis, is followed by the inhibition of glycolysis that is the only alternative pathway for ATP synthesis in anaerobic cells.
Despite the arrest of the respiratory chain, the mitochondrial membrane potential is not immediately collapsed at the onset of ischemia. In fact, the mitochondrial membrane potential is maintained by the inverse operation of the F_0F_1 ATP synthase that uses ATP generated by glycolysis. Therefore, during ischemia not only do mitochondria cease to be the major ATP producers of the cell, but they can also become its major consumers.
Prolongation of ischemia (i.e, more than 20 min of no-flow ischemia in perfused hearts) leads myocytes to a point of no return beyond which even readmission of oxygen fails to rescue cell viability. Myocardial tissue exhibits a peculiar transition from reversible to irreversible damage. In every cell type, the lack of ATP production and a severe decrease of its content result in a slow degenerative process that leads to the loss of plasma membrane integrity with release of intracellular components. Thus, if the initial pathological stimulus (i.e,

lack of oxygen) is not removed, the inhibition or the alteration of mitochondrial function results in cell death. In addition, the myocardium exhibits a peculiar transition to irreversible injury, which occurs suddenly when oxygen supply is re-established after a prolonged period of ischemia. In this case, the uncontrolled hypercontracture of myofilaments (fig. 2) is associated with a rapid increase in cell membrane permeability followed by the release of intracellular constituents.
The sudden changes produced by reperfusion on myocardial viability are concomitant with massive accumulation of calcium within the mitochondrial matrix. Intracellular Ca^{2+} overload in myocardial pathology indicates that the rapid transition toward cell death requires a coupled mitochondrial respiration. In fact, respiratory chain inhibition or uncoupling of oxidative phosphorylation blunts enzyme release occurring at the onset of post-ischemic reperfusion. Therefore, oxidative phosphorylation not only is essential for cell recovery, but can also contribute to the processes causing cell necrosis.
The apparent paradox linking mitochondrial function with cell death is related to the different ATP requirements of contraction and relaxation at the myofilament level. A suboptimal mitochondrial function could produce low levels of ATP which, in the presence of even a modest rise in $[Ca^{2+}]i$, might be sufficient for the formation of rigor bonds but not for the relaxation process, resulting in myofilament hypercontracture. Supporting this concept, at reperfusion hypercontracture is abrogated not only by preventing oxidative phosphorylation, but also by inhibiting myosin ATPase.
Regarding the causes of mitochondrial dysfunction, upon reperfusion mitochondria are exposed to high levels of Ca^{2+} that is increased in the cytosol due to both the failure of Ca^{2+} ATPases and uptake through the sarcolemma Na^{+}/Ca^{2+} exchanger. Since mitochondrial Ca^{2+} uptake and ATP synthesis utilize the same driving force, namely the Dcm, mitochondrial Ca^{2+} accumulation results inevitably in a significant decrease in ATP formation. In addition and importantly, the rise in intramitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]m$) promotes opening of the mitochondrial PTP and is associated with increased formation of ROS. Eventually a vicious cycle is generated whereby high $[Ca^{2+}]m$, PTP opening and ROS formation hamper mitochondrial function jeopardizing the maintenance of cell viability.[48, 49] PTP is a voltage-dependent, high-conductance channel located in the inner mitochondrial membrane that allows the permeability transition. This means that PTP elicits a sudden increase of the inner membrane permeability to solutes with molecular weights up to 1500 Daltons. A general consensus exists that the PTP is a major factor in determining cardiomyocyte death, and that PTP inhibition affords significant cardioprotection against the ischemia/reperfusion injury, a concept that has been successfully translated to clinical settings.[44, 47, 49, 50, 51, 52, 53, 54]
Although ROS can be generated at several intracellular and extracellular sites, in cardiac myocytes the major source is represented by mitochondria that are on the other hand a prominent target of oxidative stress. A slight generation of ROS has been observed in ischemic or hypoxic cardiomyocytes, especially in isolated cells. However, a major burst in ROS formation occurs at the onset of reperfusion providing a significant contribution to the rapid evolution toward cell death. Alterations of respiratory chain induced by ischemia are likely to contribute largely to mitochondrial ROS generation during reperfusion. However, it must be pointed out that besides respiratory chain complexes, especially I and III, also

MAOs, p66Shc, and NOX4 importantly contribute to the oxidative stress. Interestingly, pharmacological inhibition or genetic deletion of these ROS-generating systems results in a significant decrease of reperfusion-induced loss of cardiomyocyte viability.[11, 22, 23, 53]

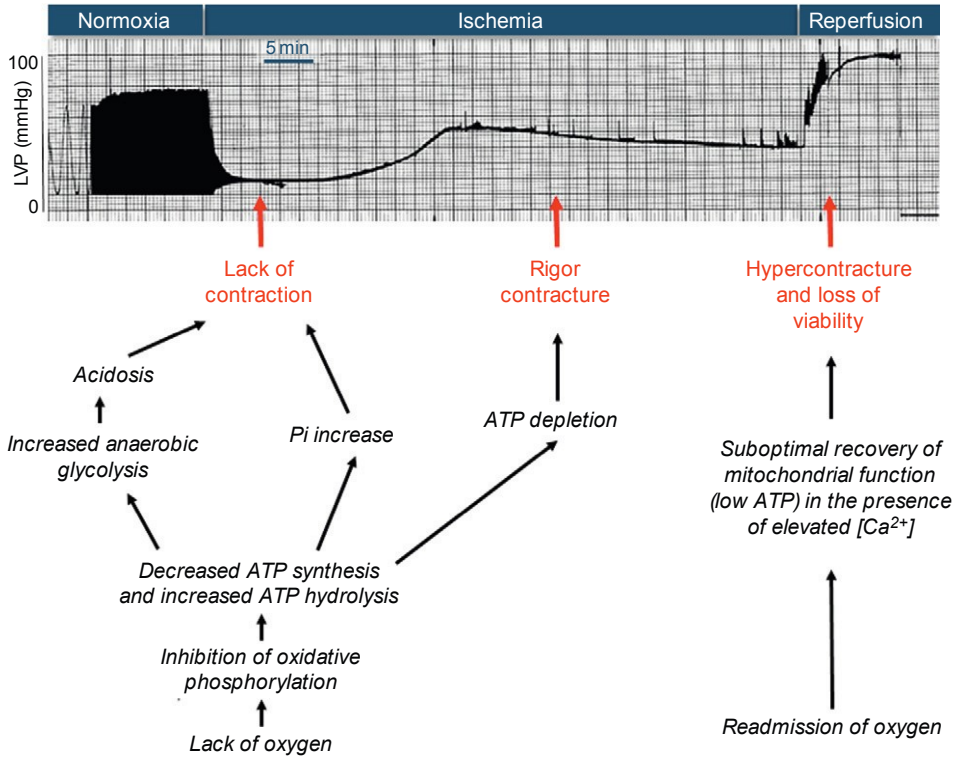

Figure 2. IR phases. *From Di Lisa, Mitochondria in Myocardial Ischemia, Encyclopedia of Biological Chemistry, 2013.*[49]

4.4.1 Inflammation in IR injury

ROS-dependent expression of pro-inflammatory stimuli and expression of adhesion molecules by endothelial cells and leukocytes promotes the infiltration and activation of neutrophils, T cells and monocytes.[55] ROS also act to enhance the inflammatory response to reperfusion via formation of oxidant-dependent proinflammatory mediators and upregulation of cytokine and adhesion molecule expression.

A broad spectrum of local and systemic mechanisms is initiated in myocardial infarction and contributes to cardiac remodelling. If uncontrolled, it may lead to negative changes in the geometry, structure and function of the ventricle and thus may have deleterious effects on cardiac function in the long term. Myocardial infarction (MI) is a condition of irreversible myocardial necrosis that results from a prolonged ischaemic insult. Owing to the limited regenerative potential of the myocardium, cardiomyocyte death elicits a reparative response that ultimately results in the formation of a scar, leading to focal contractile impairment and ventricular remodelling. Damaged cardiomyocytes in the infarcted area initiate the reparative response by releasing specific molecules, such as damage associated molecular patterns (DAMPs). These are recognized by TLRs expressed on leucocytes, parenchymal cells, and also on healthy

neighbouring cardiomyocytes. Injured cardiomyocytes release DAMPs, such as high-mobility group box 1 (HMGB1), DNA fragments, heat shock proteins, and matricellular proteins, which instruct surrounding healthy cardiomyocytes to produce inflammatory mediators. These mediators, mainly interleukin IL-1β, IL-6, macrophage chemoattractant protein (MCP)-1, and TNF-α, in turn activate versatile signalling networks within surviving cardiomyocytes and trigger leucocyte activation and recruitment.[56, 57, 58]

4.5 Inflammatory response

In the healthy organism, the innate immune system provides the first line of defense against external or internal danger signals, by initiating a protective inflammatory response. This develops during time through different phases, from initiation and full inflammation, to resolution and reestablishment of tissue integrity. The first phase of an inflammatory response is aimed at destroying pathogens, and is followed by a phase in which dead and dying cells, damaged extracellular matrix material, and cellular debris are removed, to end up with a recovery phase in which the tissue is repaired and restored to a healthy fully functional condition. In fact, if the defense against harmful threats is a priority for avoiding tissue damage, maintaining homeostasis, tissue morphology and function, is the ultimate goal of a tissue in multicellular organisms. In this perspective, inflammation evolved as an adaptive response to tissue malfunction or homeostatic imbalance. However, since the inflammatory activities are potentially harmful to the host, these need to be tightly controlled to avoid excessive tissue damage.

Pathogen-associated molecular patterns (PAMPs) together with alarmins DAMPs are different sets of molecules that alert the organism to intruding pathogens (PAMPs) or to an endogenous damage (DAMPs). PAMPs and DAMPs are recognized by cells of the innate and acquired immunity system, through toll-like receptors (TLRs) or interleukin receptor 1 (IL-1r), which are classical receptors leading to inflammatory and immune responses. In addition RAGE is another receptor that appears to play a key role in alarmin function.[59, 60]

All these receptors activate several signaling pathways. As a result, some cells are activated to destroy the pathogen and/or pathogen-infected cells, and an immunological response is triggered in order to produce and select specific T cell receptors and antibodies that are best suited to recognize the pathogen on a future occasion.[60]

4.6 Mono Amine Oxidases (MAOs)

4.6.1 Structural features

Mono Amine Oxidases are flavoenzymes that catalyze the deamination of biogenic amines. They are expressed in 2 isoforms, A and B, that display about 70% of identity. They are encoded by separate genes located on the X chromosome (Xp11.23), each comprising 15 exons with identical intron–exon organization.[111, 112]

Exon 12 codes for the covalent FAD binding site and is the most conserved exon between the two isoforms. Both A and B isoform contain the pentapeptide Ser-Gly-Gly-Cys-Tyr, where the cofactor FAD is covalently bound through a thioether linkage to the cysteine residue, namely Cys406 and Cys397 in MAO-A and –B, respectively.

This flavin moiety is the only redox factor necessary for the activity of both isoforms. MAO-A and -B are located to the outer mitochondrial membrane. They are anchored through a C-terminal helix segment that protrudes from the basal face of the structure and is highly hydrophobic, therefore facilitating the insertion into the membrane. The covalent binding of the flavin coenzyme of MAO-B allowed its identification which was resolved in the early 1970s by Singer's laboratory [113, 114] and subsequent work determined an identical structure for the covalent flavin coenzyme of human MAO-A.[115]

Both isoforms show the site of covalent FAD binding near the carboxyl terminus and the sequence site for the fold (found in most FAD-dependent enzymes) near their respective amino terminus.[116] Besides the covalent attachment of the FAD to the cysteine residues, MAO polypeptide chains undergo also post-translational modifications. The best-characterized processes are the removal of the initiator methionine in MAO-B (but not in MAO-A) and the acetylation of the N-terminus in both molecules (methionine for MAO-A and serine for MAO-B). In addition to the acetylation of the N-terminus, human, rat and mouse MAO-A and -B from several tissues have been shown to contain several acetylated lysine residues, although whether and how this modification affects MAO function remains unclear. Eventually, there are also several serine residues in both MAO-A and -B that can be phosphorylated. Among these, Ser209 inside a putative p38 consensus motif has been widely studied as a possible phosphorylation site.[117, 118] In fact, at present no definitive evidence exists for phosphorylated forms of MAO in biological systems and its putative influence on catalytic activity. Furthermore other studies documented that MAO catalytic activity does not always correlate to its level of expression, suggesting that the protein levels are not a key regulator of the enzyme activity.[117, 118, 171] MAO-B crystallizes as a dimer, with each monomer presenting a C-terminal membrane bound domain, a FAD binding domain and a substrate binding domain.[120, 120] In contrast to human MAO-B, human MAO-A crystallizes as a monomer.[122] It has only single substrate binding cavities with protein loops at the entrances of either cavity. The single cavity in MAO-A displays a rounder shape and is larger in volume than the substrate cavity in MAO-B. Analysis of residue side chains in either active site shows the substrate to have less freedom for rotation in the MAO-B site than in

MAO-A. The structural basis for this difference can be partially attributed to the conformational differences of the 200-215 residue segment that constitutes the "cavity shaping loop" in both isoforms. This loop is in a more extended conformation in MAO-A and in a more compact conformation in MAO-B.
The MAO-A and MAO-B genes are closely linked and organized in opposite directions, tail to tail 24kb apart. However, they have different promoter organizations (fig. 3).

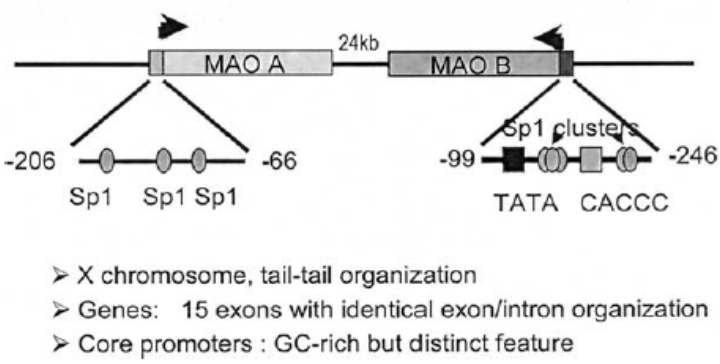

Figure 3. Schematic representation of MAO-A and –B genes and their core promoters. *From Shih et al, 2004.*[123]

The MAO-A proximal promoter consists of three binding sites for the transcription factor Sp1 in reverse orientations and it lacks a TATA box. This promoter is activated by Sp1 and repressed by a R1 (RAM2/CDCA71) repressor (fig. 3). Three glucocorticoid/androgen response elements were also found within the first 2kb of the human MAO-A promoter. The MAO-B proximal promoter contains a TATA box and two clusters of Sp1 binding sites, separated by a CACC box. It was also shown that the more distal of the MAO-B Sp1 clusters can also bind to Egr1 (Early growth response protein 1) transcription factor.[124, 123, 125, 126]

4.6.2 Tissue distribution

MAO has been localized in rodent, cat, primate, and human. It is present in most of the mammalian tissues but it differs depending on tissue nature and isoform expression.[112]
The distribution of MAO in various tissues of different species has been investigated by use of specific inhibitors, immunohistochemistry, enzyme autoradiography and *in situ* hybridization.[127, 128, 129] Brain has been intensively studied. In this organ MAO-A has been prevalently found in noradrenergic and dopaminergic neurons. Conversely, MAO-B has been detected in cell bodies of serotoninergic neurons, in histaminergic neurons and in glial cells. The distribution of MAO in the periphery varies within the same organism. MAO-A has been found in placenta, fibroblasts, liver, intestine and thyroid gland,[130, 131] while platelets, liver, pancreatic islets and kidney contain mainly MAO-B.[119, 132, 133, 134, 135, 136, 137, 138, 139]
The presence of MAO in most tissues appears to reflect a functional need. However, in some regions of the brain MAO and its substrates are not found in the same neurons. For example, MAO-A preferentially metabolizes 5-HT but it is

not found in serotonergic neurons, and MAO-B preferentially metabolizes phenylethylamine but it is present in serotonergic neurons and glial cells. The role of MAO, as previously discussed, in these regions may be to protect neurons from stimulation by oxidizing amines.[140, 141, 143]
In humans and rodents, MAO-A is present more than MAO-B in most tissues. MAO-A expression levels still remain quite the same along organism lifetime, whereas MAO-B activity increases several-fold with aging.[144, 145, 134, 146] Human cardiomyocytes contain both isoenzymes, even if MAO-A is the predominant isoform.[147] Rat cardiomyocytes prevalently contain isoform A while murine cardiomyocytes isoform B.[119, 111]

4.6.3 Biochemical role

The main biochemical role of MAO is the degradation of endogenous monoamine neurotransmitters and dietary amines by the following reaction of deamination (fig. 4):

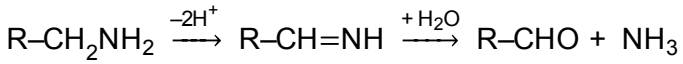

Figure 4. MAO enzymatic reaction.

This reaction occurs in two steps, ultimately resulting in the formation of the aldehyde from the corresponding amine, ammonia and H_2O_2. The aldehyde intermediate is rapidly metabolized to the corresponding acid by the action of aldehyde dehydrogenases (ALDH). By kinetic studies it has been demonstrated that the binding of the amine group to the enzyme precedes the binding of oxygen. In a first phase, the reduction of the cofactor FAD yields an aldehyde intermediate and ammonia, while in a second phase the oxidized form of the prosthetic group is restored with the concomitant production of H_2O_2 (fig. 5).

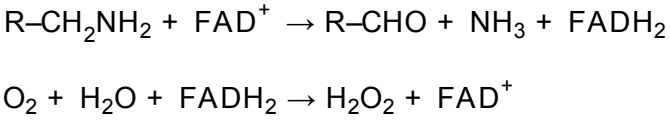

Figure 5. MAO reaction.

Aldehyde intermediates are toxic for the biological systems and a decrease in ALDH activity, also due to increased oxidative stress, might further contribute to the exacerbation of damage. H_2O_2 is a ROS that could be toxic *per se* under stress or pathological conditions, or it could generate hydroxyl radical in the presence of Fe^{2+}.[119] Together these products represent a peculiar aspect of MAO profile. However much less attention has been dedicated to them in comparison to the numerous studies on MAO activity in terminating the actions of neurotransmitters/dietary amines in central and peripheral nervous system and in the extraneuronal tissue.
MAO is the only known mitochondrial ROS source that can be inhibited pharmacologically without interfering with energy metabolism. Major advantages of investigating the role of MAO in oxidative stress are given by a defined molecular structure, specific substrates, and clinically available inhibitors.[148] MAO-A deaminates preferentially norepinephrine (NE) epinephrine and serotonin (5-HT) and it is inhibited by clorgyline. MAO-B has

major affinity for phenylethylamine, N^{τ}-methylhistamine (NMH) and benzylamine, and it is inhibited by selegiline, also known as L-deprenyl, and safinamide. Both isoforms catalyze the deamination of dopamine, tyramine, octopamine and tryptamine and are inhibited by pargyline.[112, 126, 149, 150]

4.6.3.1 MAO role in nervous system

In the central and peripheral nervous system, intraneuronal MAO-A and –B protect neurons from exogenous amines, terminate the actions of amine neurotransmitters and regulate the contents of intracellular amine stores. In the human brain, MAO-A and -B are both present in catecholaminergic cells even if the isoform A is more expressed, while MAO-B is preferentially expressed in serotonergic neurons and astrocytes.[112, 126] Given the low level of MAO-A in serotonergic neurons it has been speculated that 5-HT removal could be due to the surrounding glial cells.[112]

Extraneuronal MAO functions also in the inactivation of amines taken up by glial cells and astrocytes.[112]

4.6.3.2 MAO role in peripheral tissues

MAO in peripheral tissues, such as intestine, liver, lungs and placenta, protects the body removing amines from the blood or by preventing their entry into the circulation.[112] MAO-B in the microvessels of the blood–brain barrier presumably has a similar protective function, acting as a metabolic barrier.[112]

4.6.3.3 MAO role in heart

In the heart, MAO represents an important source of ROS, and it has been reported that it can promote and worsen oxidative stress conditions upon cardiac injuries.[29, 151] MAO-A is involved in the receptor-independent apoptotic effects of 5-HT in isolated cardiomyocytes, in post ischemic myocardial injury[152, 153] and in 5-HT-induced myocyte hypertrophy *in vitro.*[153] Regarding vasculature, it has been shown that MAO-A induces mitogenic signaling in smooth muscle cells by H_2O_2 leading to the activation of the metalloproteinase MMP-2, which likely contributes to vascular wall remodelling.[154]

4.6.4 MAO related pathologies

4.6.4.1 Loss of function

Deletion of MAO-A and MAO-B has proven their important roles in both metabolism of neurotransmitter and behavior. MAO-A knockout mice displayed elevated levels of brain 5-HT, NE and, to a lesser extent, dopamine, whereas only phenylethylamine is increased in MAO-B knockout mice. Both MAO-A and –B knockout mice show increased reactivity to stress, similar to that observed after administration of non-selective MAO inhibitors.[155]

Combined loss of MAO-A and MAO-B activity has been described in some patients with an X chromosome deletion that includes not only the Norrie disease but also the MAO-A and MAO-B genes. Furthermore, despite loss of MAO-

A and MAO-B activity, the organism survival indicates that MAO is not essential.[156]
In addition to blindness, hearing loss, and variable mental retardation that characterize Norrie disease, patients with the additional deletion of both MAO genes show profound mental retardation, autistic-like behaviour, atonic seizures, altered peripheral autonomic function, and profound alterations in biogenic amine metabolism. More recently, selective loss of MAO-A activity due to a point mutation in exon 8 of the MAO-A gene was described in Dutch kindred.[156, 157]
In this disease, selective MAO-A deficiency was associated with a clinical phenotype characterized by borderline mental retardation and impaired impulse control, including a propensity towards stress-induced aggression. Furthermore, these observations have proven that MAO-A activity is important during development even if not for the survival of the organism.
Selective deficiency of MAO-B does not lead to a specific clinical phenotype and, in particular, is not associated with clinically apparent disturbances in behaviour such as those observed in deficiencies of MAO-A. This indicates that MAO-A is considerably more important than MAO-B for metabolism of most biogenic amines in the nervous system. These functional differences in the two isoenzymes may help in understanding the basis for therapeutic interventions involving specific inhibition of one or the other isoform.[155]

4.6.4.2 Gain of function

4.6.4.2.1 Neurological diseases

In addition, MAO products, H_2O_2, ammonia and aldehydes, might have important metabolic and signaling functions in both physiological and pathological conditions, as already studied in the brain. For example, 5-HT and NE deamination leads to H_2O_2 and aldehydes production that might be involved in the regulation of sleep. However, H_2O_2 and aldehydes at higher concentrations are cytotoxic.[158, 159, 160] The aldehyde derived from dopamine, which does not appear to accumulate in the healthy brain, is cytotoxic in condition of neurodegenerative pathology. This might be important in Parkinson's disease, in which the levels of ALDH in the substantia nigra are greatly reduced.[112] In addition, MAO-dependent H_2O_2 overproduction contributes to the degeneration of nigral cells in Parkinson's disease. In Parkinson's disease MAO inhibition or iron chelation achieves the same neuroprotective effect, strongly suggesting that a reduction of local oxidative stress is a major component of this neuropathology.[161]
Other studies have reported that MAO-B isoform is increased in the brains of patients with Parkinson's disease. Thus MAO-B inhibitors such as l-Deprenyl and safinamide were investigated as adjuvants to l-DOPA, the aminoacidic precursor of NE and dopamine already used in the treatment of Parkinson. Those studies reported l-Deprenyl and Safinamide are efficient both as adjuvants to the treatment with l-DOPA. Furthermore, l-Deprenyyl is effective also as monotherapy.[162]
Neurodegenerative diseases, such as Huntington's disease and amyotrophic lateral sclerosis (ALS), share many of the pathological features of Parkinson's disease and Alzheimer's disease, such as oxidative stress, iron accumulation, excitotoxicity, inflammatory processes and the misfolding of toxic proteins that

cannot be degraded after ubiquitination. Regarding Alzheimer's disease, lot of studies are focused on the design of new MAO inhibitors, starting from the structure of selegiline, propargylamine and coumarin derivatives.[163, 164, 165, 166] At the moment, only rasagiline, a specific MAO-B inhibitor, has been reported to be effective in mouse models of ALS, indicating the need of further studies to assess MAO inhibitor as eventual and successful drugs in these conditions.[112]

4.6.4.2.2 Tumors

Some papers also reported a link between MAO and tumor growth. It has been published that MAO-A is significantly downregulated in hepatocellular carcinoma (HCC) by epigenetic alteration and that its expression is closely associated with the key events of tumor metastasis.[167] Conversely, MAO-A expression is increased in human glioma tissue specimens and cells, wherein clorgyline, MAO-A inhibitor, is effective in reducing tumor growth and cell survival.[168] MAO-A is also increased in Prostate Cancer cells and it promotes HIF1α/VEGF-A/FOXO1/ TWIST1 pathway that enhances tumor growth.[169]

4.6.4.2.3 Muscular diseases

MAO-A-mediated oxidative stress can also lead to cell damage, representing a novel pathogenetic mechanism for glucocorticoid-induced myopathy and a potential target for therapeutic intervention. MAO-A expressed in response to glucocorticoid administration plays an important role in glucocorticoid-induced toxicity in skeletal myocytes by producing H_2O_2.[170] MAO-dependent oxidative stress is also involved in myofiber damage. Indeed, it has been demonstrated that MAO inhibition is able to reduce the occurrence of myofibrillar protein oxidation and myofiber defects in two murine models of muscular dystrophies (MDs). In addition, besides reducing biochemical and histological alterations, MAO inhibition ameliorates contractile performance highlighting the relevance of mitochondrial ROS formation in MDs.[171]

Pargyline treatment was shown to significantly reduce both ROS accumulation and mitochondrial dysfunction, and to normalize the increased incidence of apoptosis in myoblasts from patients affected by collagen VI myopathies. Those observations indicated that MAO-dependent oxidative stress is causally related to mitochondrial dysfunction and cell death also in human myoblasts.[172]

4.6.4.2.4 Heart diseases

MAO activity affects also cardiac biology and function with different possible mechanisms.[119, 173] MAO-dependent ROS formation contributes to the apoptotic cascades induced by IR.[29] Indeed MAO substrate 5-HT-derived ROS have been shown to promote apoptosis in IR-associated events, such as myocardial dysfunction. The production of MAO proapoptotic ROS is linked to ceramide increase but also to the fall in the intracellular antiapoptotic S1P content caused by a decline of Sphingosine Kinase 1 (SphK1) protein and activity. SphK1 overexpression markedly inhibits ROS-mediated, serotonin-induced cardiomyoblast apoptosis. Enforced SphK1 expression reduced ceramide elevation (triggered by 5-HT) by driving ceramide metabolism toward the synthesis of S1P. MAO mediates SphK1 activity and changes the ceramide/S1P

sphingolipid balance promoting cardiomyocyte apoptosis.[152, 174]
Blockade of endogenous MAO activity reduces also the vascular formation of H_2O_2 and partially restores normal endothelium-dependent relaxation in vessels pre-exposed to Ang II or LPS.[175]
A study by Villeneuve and colleagues showed that cardiac-specific overexpression of MAO-A was accompanied by ultrastructural defects of cardiac mitochondria, ATP depletion and ultimately led to cardiomyocyte necrosis and heart failure.[176] Our laboratory demonstrated also that enhanced MAO-A activity coupled with increased intramyocardial NE availability results in augmented ROS generation. This pairing contributes to maladaptive remodeling and left ventricular dysfunction in hearts subjected to chronic stress.[177] Furthermore MAO inhibitors improve cardiac function in patients with heart failure.[119]

4.7 Macrophages

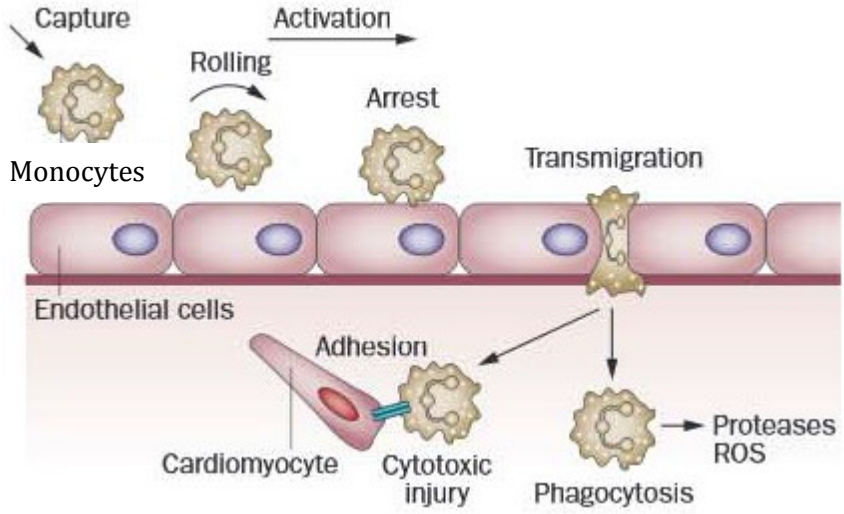

Figure 6. Cell recruitment upon inflammation. *From Frangogiannis, 2014.*[57]

During the initial phases of an inflammatory reaction, there is the recruitment of leukocytes (fig. 6). These cells are represented by different cytotypes that contribute to the inflammatory response, such as monocytes (fig. 6), dendritic cells, macrophages, lymphocytes.
Circulating monocytes can be recruited by tissue resident macrophages, lymphocites alongside with other tissue cells in the site of inflammation where they differentiate depending on the environmental stimuli. Monocytes are plastic cells able to differentiate in two main subtypes: dendritic cells and macrophages. Dendritic cells are also defined antigen-presenting cells. Their task is to process antigen molecules to present them on their surface to T cells. Macrophages (MΦ) are phagocytic cells that are classified in many groups. [61, 62, 63, 64, 65, 66, 67] We will focus on the two main representing classes: M1 and M2. M1 MΦ (or classically activated macrophages, CAMs) are pro-inflammatory and have potent microbicidal and tumoricidal activity, whereas the M2 MΦ (or alternatively activated macrophages, AAMs) are involved in tumor progression and tissue remodeling, including fibrosis.[67]

4.7.1 Macrophage differentiation

4.7.1.1 Resident MΦ

The so-called resident MΦ can be heterogeneous cells and they derived from hematopoietic precursors or from primitive cells of the yolk sac. They can either persist in an organ by local proliferation or depend on monocyte recruitment for repopulation. Once they reached the tissue, macrophages appear to retain their plasticity and not only change their own physiology, but also alter the physiology of cells adjacent to them.[68, 67]

4.7.1.2 Monocyte derived MΦ

Monocytes differentiate in macrophages responding to different stimuli, and these are the classically used: granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF, also known as CSF-1). GM-CSF and M-CSF drive the monocyte/macrophage development, differentiation, and proliferation.[69, 70]

GM-CSF is a glycoprotein that acts through its specific receptor, called Csf2rα,[71, 72] to promote M1 polarization (fig. 7). It activates a signaling pathway constituted by IFNγ-mediated Janus kinase–signal transducer and activator of transcription (JAK–STAT) that induces IFN regulatory factor (IRF5) transcription. High expression of IRF5 is characteristic of M1 macrophages, in which it directly activates transcription of the genes encoding interleukin 12 and IL-23 and represses the gene encoding IL-10.[70, 73, 74, 75]

The biological effects of M-CSF molecule are mediated by its unique receptor (M-CSFR), a member of the tyrosine kinase receptor family encoded by the c-fms proto-oncogene, which is prominently expressed in cells of the monocyte/ macrophage lineage.[76, 77] Binding of M-CSF to the extracellular domain of the M-CSFR leads to receptor autophosphorylation on specific tyrosine residues that serve as anchoring sites for signaling molecules (fig. 7). Therefore, activated M-CSFR plays a pivotal role in the assembly of the specific multimeric complexes that will physically organize downstream signaling pathways, including the PI 3-kinase/Akt pathway and the Ras/Raf/MEK/MAPK pathway to eventually promote M2 differentiation.[78 79, 80, 81]

4.7.2 Macrophage polarization

The second step in macrophage development is linked with their polarization. Macrophage polarization occurs through different activation programs by which macrophages carry out their defensive functions (fig. 7). In this way, macrophages become able to respond with appropriate functions in distinct contexts. Essentially, macrophages can still modify their metabolic functions from a killing/inhibitory capacity (M1 MΦ), to a heal/growth promoting setting (M2 MΦ).[69]

M1 activation commonly requires priming with IFN-γ, the canonical cytokine generated by Th1 cells, lipopolysaccharide (LPS) (used in this work), a plasma

membrane component of Gram-negative bacteria, and activation of the downstream transcription factors, such as STAT1, nuclear factor-kappa light chain-enhancer of activated-B cells (NF-κB), and IRF-5, as already reported during M1 differentiation process. These M1 macrophages express inflammatory genes, including tumor necrosis factor α (TNF-α), IL-1β, and IL-6.
M2 macrophages are activated by Th2 cytokines, IL-4 and/or IL-13.
M2 polarized MΦ produce IL-10, IL-1 receptor antagonist (IL-1ra), and transforming growth factor-β (TGF-β), that attenuate inflammation and promote extracellular tissue remodeling. Transcription factors involved in M2 polarization, as during differentiation, include STAT3, STAT6 and IRF-4, and peroxisome proliferator-activated receptor (PPAR)-γ.[70]
M1 and M2 MΦ have distinct features in terms of chemokine production profiles, and iron and glucose metabolism. M1 show an elevated activity in the pentose phosphate pathway, and an elevation in certain Krebs cycle intermediates, including succinate and citrate. M1 macrophages also have increased synthesis of fatty acids and all these metabolites can act as signals to alter M1 cell function. In contrast, metabolism in M2 macrophages comprises high levels of fatty acid oxidation and oxidative phosphorylation.[69, 70, 81]

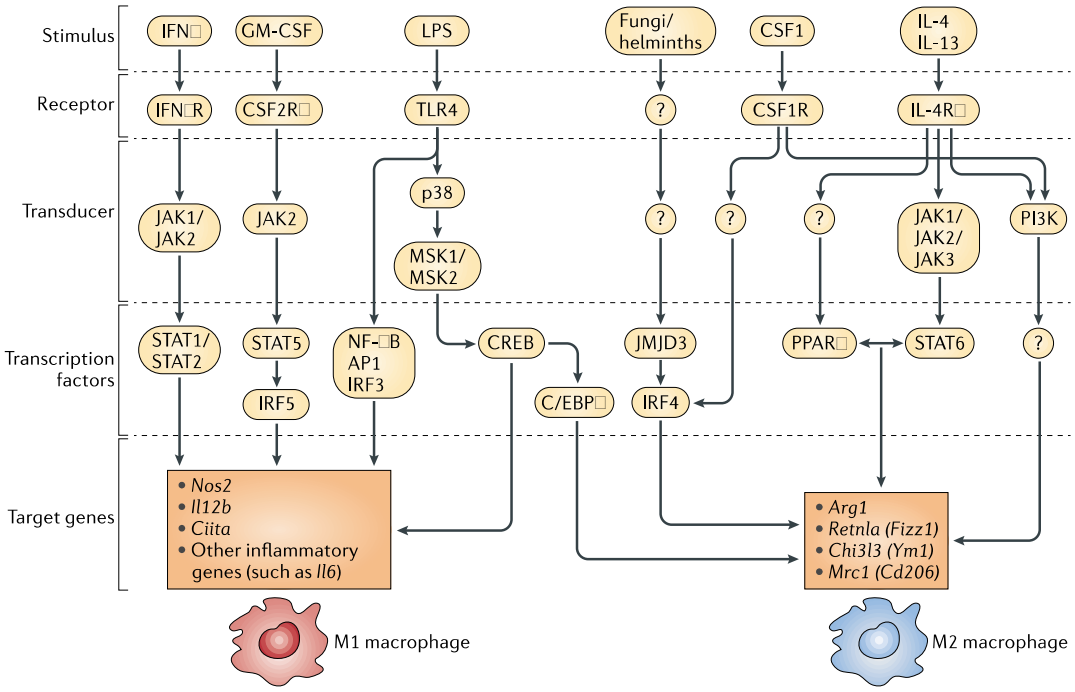

Figure 7. Main signal transduction pathways to M1 and M2 MΦ differentiation/polarization. *From Lawrence and Natoli, 2011.*[70]

4.7.3 Mitochondria in phagocytic cells

Therefore, mitochondria in phagocytic cells can no longer be viewed solely as the energy machinery but as a vital source of dynamic signals that respond to the surrounding environment. LPS activation stimulates the production of citrate, succinate and ROS, all of which act in a pro-inflammatory manner to fight infection. On the other-hand, IL-4 coerces macrophages towards high levels of fatty acid oxidation and oxidative phosphorylation, both of which are essential

for longer-term survival of macrophages required for their roles in wound repair and resolution of infection.
We can therefore directly map the mitochondrial phenotype of macrophages to their specific effector functions, which has exciting therapeutic prospects. It is possible that manipulating the metabolism of macrophages could boost immune responses, or indeed dampen inflammatory responses in the case of auto-immunity.[70, 81]
Furthermore, it has been demonstrated the involvement of mitochondria in LPS-induced ROS signalling; (i) to propose the mitochondrial protein UCP2 as a key regulator of this process; and (ii) to provide mechanistic evidence to explain the immune phenotypes of $UCP2^{-/-}$ and $UCP2^{+/+}$ macrophages.[83]

4.7.4 ROS regulate macrophages

The role of oxidative stress in macrophage metabolism is controversial and still under debate.
Several roles of mitochondrial ROS production have been identified in M1 macrophages. Firstly, ROS is directly bactericidal. LPS signaling induces tumor necrosis factor receptor-associated factor 6 (TRAF6) recruitment to evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) in the outer mitochondrial membrane, where it interacts directly with complex I of the respiratory chain, promoting its assembly and subsequent ROS production.[84] The bactericidal ROS enters phagosomes which contain the engulfed bacteria and promotes the clearance of infection. Second, ROS can influence the production of pro-inflammatory cytokines, including IL-6 and TNF-α.[85] ROS prevent the dephosphorylation of mitogen activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK phosphorylation in response to LPS, which may contribute to enhanced cytokine production. Treatment with MitoQ, the specific mitochondrial ROS scavenger, also significantly reduced LPS-induced IL-6, TNF-α and IL-10 in human PBMCs; this may be a result of decreased MAPK activity.
Thirdly, mitochondrial ROS has been shown both to activate and prime the nucleotide-binding domain and LRR-containing NLR (NLRP3) inflammasome, which is a protein complex required for the production of mature IL-1β.[86, 87, 88]
Also M2 macrophage differentiation and polarization are driven by ROS. Indeed, M-CSF induced production of ROS *per se* in monocyte differentiation to M2 MΦ, as it is required for a physiologic response, such as cell proliferation. It has been demonstrated that oxidative inactivation of the redox-sensitive Src homology region 2 domain-containing phosphatase 1 (SHP1) specifically might promote the PI3K/Akt signaling pathway specific for M2 differentiation. Furthermore, the exogenous addition of both H_2O_2 and M-CSF leads to phosphorylation of Akt in addition to MAPKs, including ERK, p38, and JNK, therefore simulating the M2 differentiating pathway.[89] On the other hand, the exacerbation of the oxidative stress conditions due to elevated $O_2^{\bullet-}$ levels, lead to increased phosphorylation of stress-responsive MAPKs, p38, and JNK-1, inciting monocyte activation.[90, 90]
It has been demonstrated that the antioxidant BHA blocks IL-4/ IL-10-triggered polarization to human and murine M2 MΦ, but did not affect LPS/IFNγ-induced polarization to M1 MΦ. These data demonstrate that elimination of ROS during

MΦ differentiation only blocked the differentiation to M2, but not to M1 MΦ.[92] Other studies reported various effects of different antioxidants during MΦ polarization. Catalase antioxidant activity was ineffective on human macrophages, while diphenyleneiodonium (DPI) or N-acetylctsteine has demonstrated the capacity to diminish the IL-10 and IL-12 production of the M2 subsets, although it showed no effect on IL-6 production.[93, 94]
Furthermore, ROS are directly requested to modulate autophagy. ROS regulates autophagic signaling transduction pathways that induce autophagy such as mTOR and MAPK and these evidences were clear by the use of anti-oxidant.[94] Another report showed that most monocytes do not survive when autophagy is blocked during differentiation, especially when blocked in its early stages, supporting the pivotal role for ROS in macrophage differentiation.[95]

4.7.5 Macrophages in cardiac injury

It is well known that cardiac injury leads to an inflammatory response, as previously described also in paragraph 4.4.2. After myocardial injury, the number of macrophages increases, which is important for the progression and resolution of tissue injury.[68] Initial work suggested that the early phase after infarction recruits inflammatory M1 MΦ [96, 98, 99,] that, by exercising inflammatory functions, are potentially harmful in this early phase, as they promote myocardial damage.[100] On the other hand, M2 MΦ could promote angiogenesis, extracellular matrix synthesis and myocardial healing through secretion of anti-inflammatory cytokines and growth factors, like VEGF and TGF-β.[96, 98, 101]

4.7.6 MAO in macrophages

To date the literature related to MAO in macrophages has focused the attention only on its cellular expression and on its function of immune transmitter removal, such as 5-HT. 5-HT is involved in interactions between the central nervous and immune systems through 5-HT_{1A} receptor. These include T cell and natural killer cell activation, delayed-type hypersensitivity responses, production of chemotactic factors, and natural immunity delivered by macrophages.[103, 104, 105]
In human peripheral blood monocytes, it has been reported that IL-4 and IL-13 up-regulate expression of a variety of gene products. Among those genes MAO-A presents a 55-fold increase.[106, 107] The up-regulation of MAO-A is likely to proceed via 15-lipoxygenase (15-LOX1)-dependent and -independent pathways as already published. 15-LOX1 is a pro-oxidative enzyme involved in the production and metabolism of fatty acid hydroperoxide. U937 cells transfected with 15-LOX1 or incubated with primary 15-LOX1 products (hydroperoxy fatty acids) or H_2O_2 showed MAO-A increased expression levels, whereas the corresponding hydroxy fatty acids on the same cytotype were ineffective.[108] According to these findings, other papers indicate that Janus Kinases (Jaks), -non receptor tyrosine kinases that transduce cytokine-mediated signals via the JAK-STAT pathway-, which are commonly required for LOX induction, are involved also in MAO expression. Indeed it has been demonstrated that IL-4 and IL-13-

activated monocytes induce MAO-A expression through Jak1 and Jak2 upstream regulation, respectively.[109]

Interestingly tyramine and benzylamine, MAO substrates, elicited a dose-dependent decrease of LPS/INF-γ-induced nitric oxide synthase (NOS2) expression in rat peritoneal macrophages. Pargyline, a MAO specific inhibitor antagonized the negative effect of these substrates on NOS2 expression. These data demonstrated that MAO-generated H_2O_2 is an important inhibitor of inducible NOS2 expression. Although NOS2 generation of NO is differently regulated in murine and human macrophages, it has been speculated that the expression of MAO-A in M2 macrophages may stabilize the M2 anti-inflammatory phenotype in part by preventing the expression of NOS2 and generation of NO.[110, 105]

5. Materials and methods

5.1 Cell cultures

5.1.1 Neonatal Rat Ventricular Myocytes (NRVMs)

Littermate Wistar rats, provided by the Department of Biomedical Science in the University of Padova, were sacrificed by cervical dislocation. Neonatal rat ventricular myocytes (NRVMs) were isolated from P1-3 pups. Hearts were collected, washed and excised into pieces and then incubated o.n. at 4°C in Hank's Balanced Solution (HBSS – Sigma) containing 2.5% Trypsin 10X (Thermo Fisher Scientific). The day after heart fragments were washed and further dissociated with a solution of HBSS and type II collagenase (Thermo Fisher Scientific) for 5 times, under continuous stirring. Cell suspension was filtered with a 40μm strainer and pelleted. After that cells were plated for 90'. After this incubation, only fibroblasts are attached to the flask, while myocytes remain in the supernatant. Then cell suspension was counted and plated over plates or coverslips previously coated with a solution of 0.1% of porcine gelatin (Sigma).
Cells were grown in modified essential medium (MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 10 units/ml penicillin, 100μg/ml streptomycin (Thermo Fisher Scientific), 5-bromo-2'-deoxyuridine (BRDU, Sigma), 0.1% Non essential aminoacids (NEAA, Gibco-Thermo Fisher Scientific) and 1X Insulin Transferrin Selenium (ITS, Thermo Fisher Scientific). Cells were plated at a concentration of $5x10^5$ cells/ml, resulting in 80-90% confluence, and are cultured for 24h in a 5% CO_2 incubator at 37°C prior to the experiments.

5.1.2 Human Macrophages (MΦ)

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donor (hospital center, Padova) and isolated using a Ficoll (Thermo Fisher Scientific) and Percoll (GE Healthcare) density gradient (Fluks, 1981; Almeida et al. 2000). Blood was centrifuged to separate cell suspension from serum and then washed with PBS 1X (Thermo Fisher Scientific). After that linfo-monocytes were isolated stratifying the suspension over an equal volume of Ficoll and centrifuged 2000 rpm 30' at room temperature without accelerator and decelerator. A second centrifuge was required to purify monocytes from lymphocytes taking advantage of a second gradient due to Percoll. At the end cell suspension was plated for 1h into 24-well culture plates at a density of $2x10^6$ cells/well in RPMI without serum to allow monocyte adhesion and then washed 3 times with Roswell Park Memorial Institute 1640 Medium I to eliminate residual lymphocytes (RPMI - Thermo Fisher Scientific).
Subsequently RPMI containing 20% FBS, 10ng/ml macrophage colony stimulating factor (M-CSF, Miltenyi), or 10ng/ml granulocyte macrophage colony stimulating factor (GM-CSF, Peprotech) was added to monocytes to induce differentiation to macrophages (Thermo Fisher Scientific), After 5 days of

incubation at 37°C, 5% CO_2 half of the medium was replaced with a fresh one, and after 7 days of macrophage differentiation, cells were treated for different time periods (1-2-3-24h) to polarize them as described at 5.2.

5.1.3 RAW 264.7 murine cell line

Macrophage RAW 264.7 cells were cultured in RPMI 1640 with 10% FBS, 10 units/ml penicillin, 100µg/ml streptomycin at 37°C in 5% CO_2. Prior to treatment, cells were plated in 24 well culture plates at a density of $2x10^6$ cells/well and cultured for 24h.

5.1.4 Bone Marrow Derived Macrophages (BMDM)

Mice were sacrificed through cervical dislocation. Total bone marrow was obtained from mice by flushing femurs and tibiae with Iscove's Modified Dulbecco's Medium (IMDM - Thermo Fisher Scientific). Cell suspension was incubated for 5' at room temperature with ACK (Ammonium-Chloride-Potassium) Lysis Buffer, used for the lysis of red blood cells, and then white cells were pelleted.
Bone marrow mononuclear phagocytic precursor cells were propagated in suspension by culturing in macrophage medium IMDM containing 10% FBS (Thermo Fisher Scientific), 100units/ml penicillin and 100µg/ml streptomycin (Thermo Fisher Scientific), supplemented with 10ng/ml M-CSF (Miltenyi), or 10ng/ml GM-CSF (Miltenyi) in tissue culture plates (Stanley, 1997). The cells were maintained at 37°C in a humidified incubator with 5% CO_2.
After 3 days half of the medium was replaced with a fresh one and after 7 days macrophages differentiated for the experiments were treated for different time period (24-48h) to polarize them.

5.2 Macrophage treatment

Human and murine MΦ were incubated for different time point (3, 6, 24h) with LPS 1µg/ml (Sigma) or IL-4 20ng/ml (Miltenyi) in combination with IL-13 10ng/ml (Miltenyi) and MAO inhibitor Pargyline (100µM Sigma). To verify the involvement of MAPK p38 and transcription factor Sp1 in MAO expression, cells were incubated for 30' before LPS and cytokine treatment with i) SB202190 10µM, a specific p38 inhibitor and ii) mithramycin 5µM (Sigma), a specific inhibitor for Sp1 activation. All the used cytokines were specific for mouse or human samples.

5.3 Denervation treatment

Mice were injected intraperitoneally with 0.1mg/g mice weight 6-hydroxydopamine-hydrochloride (6-0H-DOPA - Sigma) (Finch et al, 1973). The animals were sacrificed after 24h and their hearts were isolated and subjected to 2 different protocol of oxidative stress. After the treatments hearts were frozen

for the following analysis. The chemical denervation was assessed through western blot analysis for every treated heart and for this purpose tyrosine-hydroxylase antibody (Sigma) was used as typical marker to target synaptic terminals.

5.4 Treatment protocols of oxidative stress

Adult (aged 4 months) male C57BL/6 WT mice (Charles River, Padova, Italy) were sacrificed by cervical dislocation. Hearts were perfused with bicarbonate buffer gassed with 95% O_2–5% CO_2 at 37°C (pH 7.4) at a constant flux of 5 ml/min. Perfusion was performed in the nonrecirculating Langendorff mode. The perfusion buffer contained (in mM) 118.5 NaCl, 3.1 KCl, 1.18 KH_2PO_4, 25.0 $NaHCO_3$, 1.2 $MgCl_2$, 1.4 $CaCl_2$ and 5.6 glucose. Hearts were treated as follows (n ≥ 6/group): after 10' of stabilization, hearts were subjected to i) 15' of perfusion with 1mM H_2O_2; ii) 40' of global ischemia by stopping the coronary flow and 15' of reperfusion. After these treatments, hearts were frozen in liquid nitrogen and stored at −80°C for analysis. Treatments were performed adding the MAO inhibitor pargyline (0.5 mM) to the perfusion buffer.

5.5 Measurement of lactate dehydrogenase activity

To determine the amount of lactate dehydrogenase (LDH) released from the hearts exposed to H_2O_2 or IR protocol, coronary effluent was collected at 1' intervals during the 15' of reperfusion as previously described. At the end of reperfusion hearts were collected and homogenized for assessing the residual activity of LDH in the whole tissue.
Since all values were normalized to heart weight, the amount of LDH released was expressed as % of total (i.e, effluent + homogenate) to rule out possible changes due to variations in heart size. LDH activity was determined by spectrophotometrically measuring the absorbance of NADH at 340nm (reduction of pyruvate to lactate) according to the following reaction (fig. 8).

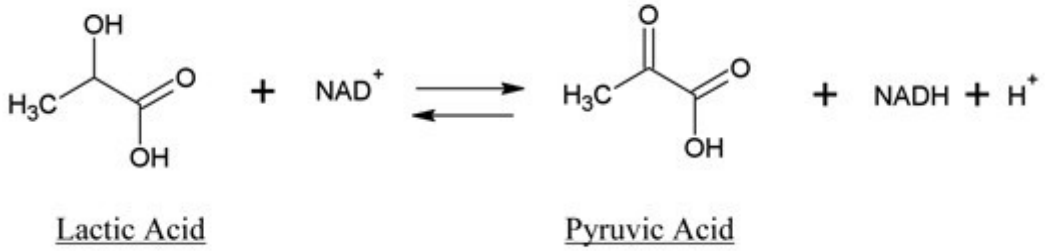

Figure 8. Lactate dehydrogenase reaction.

5.6 Mass Spectrometry (MS) analysis

Liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) of metabolites in heart samples and neonatal rat ventricular myocytes were performed in collaboration with Dr. Alessandra Castegna (University of Bari, Italy).

5.7 Fluorescence microscope

5.7.1 Measurement of oxidative stress

ROS concentration was measured using MitoTracker® Red CM-H2Xros (MTR, Thermo Fisher Scientific, λ_{exc} = 579 nm, λ_{em} = 599 nm) probe and the cationic dihydroethidium probe (DHE, Sigma, λ_{exc} = 518 nm, λ_{em} = 605 nm).
MTR is a reduced dye, that passively diffuses across the plasma membrane and accumulate in active mitochondria. It does not fluoresce until it enters live cells, where it is oxidized by reactive oxygen species (ROS). MTR contains a mildly thiol-reactive chloromethyl moiety for labeling mitochondria The cells were seeded on 24mm coverslips and incubated for 30' at 37°C with 20nM MTR in HBSS.
DHE freely permeates cell membrane and when it is oxidized, mainly by superoxide anion, it becomes red fluorescent. Oxidized DHE binds to nuclear DNA, a process that results in a further increase in fluorescence.
Heart slices were incubated for 30' at 37°C with 5µM DHE in degassed PBS and then washed twice in PBS 1X. Considering that the solution is photosensible, all the operations were carried out in dark.
For each analyzed coverslip DHE fluorescence intensity was calculated as the mean fluorescence of all the selected ROIs.
Images were collected using a confocal microscope (Leica SP5), a 63X oil-immersion objective and appropriate emission filters.

5.8 Cytofluorimetric Analysis

5.8.1 ROS measurements

Cells were loaded with DCFDA 10µM (2′ ,7′ -Dichlorofluorescin diacetate Sigma) for 30' at 37°C. Cells were collected and centrifuged for 5' at room temperature at 1500 rpm. Cells were resuspended in FACS buffer (PBS 1X, 1%FBS) and 10,000 events/sample were analyzed using a FACSCanto® analyzer (Becton Dickinson). Dead cells were excluded from FACS analysis by adding propidium iodide (PI).
Data were processed using the FACSDiva software.

5.8.2 Marker expression

Viability and purity of macrophages were determined by flow cytometry analysis (BDFacsCanto, Becton Dickinson). Viability was assessed by staining the death cells with PI. The cell population negative for PI was analyzed further for CD68 expression (>99%) a general marker for the total macrophage population. CD80 marker was used to assess the correct expression of the pro-inflammatory M1 MΦ, while CD163 marker was used for the anti-inflammatory M2 ones.
Cells were detached and resuspended in FACS buffer, divided in different samples and incubated with CD68, CD86 and CD163 labelled antibodies (Beckton Dickinson) for 30' at 4°C. At the end samples were washed, centrifuged for 5' at room temperature at 1500rpm and resuspended in FACS buffer. The analyses were performed using the BDFACSDiva software.

5.9 ELISA assay

MΦ supernatants were collected after 7 days of plating and after the treatments to be tested in sandwich ELISA (Peprotech and Beckton Dickinson), according manufacturer's instruction.

5.10 Measurement of hydrogen peroxide formation and MAO activity

Hydrogen peroxide formation was determined using Amplex Red. The assay is based on the detection of hydrogen peroxide generated during substrate catabolism in a horseradish peroxidase (HRP) coupled reaction (fig. 9) using 10-acetyl- 3,7-dihyrdoxyphenoxazine (Amplex Red reagent, Molecular Probes).

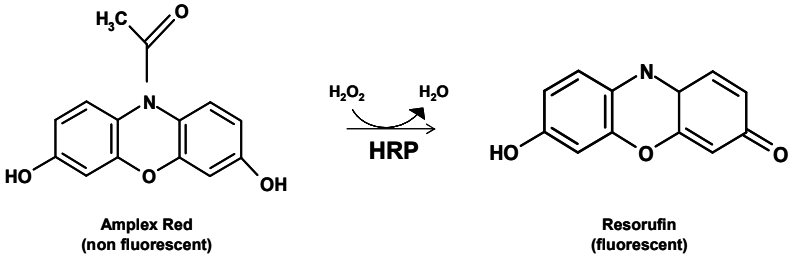

Figure 9. Hoseradish peroxidase reaction.

For the measurements of hydrogen peroxide production from MAO activity the isolated enzyme was incubated in a standard buffer (KCl 120mM, MOPS/TRIS 20mM, pH 7.4) in the presence of 5μM Amplex Red reagent and 4mg/ml HRP.
The reaction was started adding 10, 50 or 100μM of N^{τ}-methylhistamine, histamine and tyramine as positive control. Fluorescence intensity was recorded for 10' at 37°C using a Perkin Elmer LS-50B fluorimeter at the 544/590nm excitation/emission wavelengths. Parallel samples were run in the absence of substrate determine an eventual increase in fluorescence not due to MAO activity.

5.11 Cell extracts and western blot analysis

5.11.1 Sample preparation

$2x10^6$ cells for well were seeded in a 24-well plate and collected after the treatment. Cells were resuspended in 100µL of the following Lysis Buffer: NP-40 1%, sodium deoxycholate 0.25%, EDTA 2mM, SDS 2%, 1X protease and 1X phosphatase inhibitor mix (Roche) in PBS 1X. Samples were homogenized by mechanical passages through the pipet and their protein concentration was determined with Bradford method (BioLegend). Then 33µL of Sample Buffer 4X (glycerol 20%, SDS 3%, Tris 75mM, bromophenol blue 0.02%, β-mercaptoethanol 5% in H_2O, pH 6.8) were added and the samples were heated at 100°C for 10'. Finally they were loaded on the gel or stored at -20°C.

5.11.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

Electrophoresis was performed on polyacrylamide gel prepared in glass labs 0.75mm thick with 12% acrylamide in the separating gel and 5% in the stacking gel. The following solutions were used for the preparation of the gel and the electrophoresis run:

Acrylamide/bisacrylamide: 30% acrylamide and 0.8% bisacrylamide
Lower Tris-HCl (4X): 1.5 M Tris-HCl and 0.4% SDS, pH 8.8
Upper Tris-HCl (4X): 0.5 M Tris-HCl and 0.4% SDS, pH 6.8
Running buffer (4X): 0.1 M Tris-HCl, 0.77 M glycine and 0.4% SDS, pH 8.3

Gel polymerization was obtained by the addition of TEMED (Applichem) and ammonium persulfate 0.1mg/ml (Applichem). Samples were run on the gel at room temperature using an Electrophoresis Power Supply (Apelex) that provided a constant voltage of 70V in the stacking gel and 150V in the separating gel.
In order to make the proteins accessible to antibody detection, they were moved from the gel onto a nitrocellulose membrane. A 0.45µm nitrocellulose membrane (BioRad Laboratories) was placed on top of the gel, avoiding bubbles, and placed on top. This sandwich was then inserted into a transfer box filled with Transfer Buffer, so that the gel is oriented towards the cathode and the membrane towards the anode. When a current is applied to the electrodes, this causes the proteins to migrate from the negatively charged cathode to the positively charged anode, i.e. towards the membrane. Proteins were transferred for 18h at 150mA at 4°C.
Once the transfer was carried out, the membrane was saturated with fat-free milk 5% in TBS (Tris-HCl 50mM, NaCl 150mM, pH 7.5) Tween 0.1% for 1h at room temperature. The antibodies used to detect the proteins of interest were diluted in milk 0.1% or BSA 3% in TBS-Tween 0.1%. The following primary antibodies were used:

Rabbit Anti-MAO-A (AbCam), dilution 1 : 1000
Rabbit Anti-MAO-B (Sigma), dilution 1 : 1000
Mouse Anti β-actin (Sigma), dilution 1 : 5000
Rabbit Anti histidine-decarboxylase (Sigma), dilution 1:500
Rabbit anti-tyrosin-hydroxilase (Sigma), dilution 1:1000
Rabbit anti-Phospho-p42/44(Erk) (Cell signaling) 1:2000
Rabbit anti-p42/44(Erk) (Cell signaling) 1:1000

All the primary antibody incubations were carried out overnight at 4°C upon gently shaking. Following the incubation, membranes were washed 3 times for 10' with TBS-Tween 0.1%. Secondary antibodies were diluted in TBS-Tween 0.1% and incubated with the membrane for 1h at room temperature. Fluorochrome ‐ conjugated secondary antibodies used were:

Rabbit Anti-Mouse (LICOR), dilution 1 : 10000
Mouse Anti-Rabbit (LICOR), dilution 1 : 10000

Finally membranes were washed 3 times for 10' with TBS 1X and detection was performed with the Odyssey Infrared Imaging System (LICOR Biosciences).

5.11.3 Densitometry

Images of the acquired western blots were analyzed using the Image J software. This program allows the quantification of the optical density of bands or dots that is directly proportional to the protein content.

5.12 Statistics

Results are presented as mean ± SEM. When the experiment was repeated more than 3 times, comparisons between two groups of data were performed via 2-tailed unpaired Student t test. A P-value less than 0.05 was taken to indicate a significant difference that was then tagged with an asterisk.

6. AIMS OF THE STUDY

The first aim of this study was to clarify the mechanisms that induce MAO activation in conditions of oxidative stress and cardiac injury. The increased MAO activity can be attributed to (i) an increase in its expression, (ii) post-translational modifications and/or (iii) increased availability of substrates. However, cardiac MAO expression hardly changes in several pathophysiological conditions and covalent modifications cause just minor changes of their activity. Therefore, to test the third hypothesis, identification and quantification of MAO substrates were performed in *ex vivo* and *in vitro* model of cardiac injury, i.e. Langendorff-perfused mouse hearts and neonatal rat ventricular myocytes, respectively. Two different protocols of oxidative stress were set up in the *ex vivo* model. Mass spectrometry (MS) analyses, ROS measurements and viability assays allowed to identify and quantitate for the first time a range of metabolites involved in these protocols. Our attention was focused on the most abundant substrate of MAO that was found in our experimental models, N^{τ}-methylhistamine (NMH), which originates from histamine methylation and represents the main product of its intracellular catabolism. The potential sources of histamine were characterized, considering both myocyte and non-myocyte origin. To this aim synaptic terminals were considered as non-myocyte sources and we excluded them by a protocol of chemical denervation. Then cardiomyocyte was analysed as possible source in the *in vitro* model. Furthermore, we investigated the mechanisms underlying histamine involvement in MAO activation in isolated cardiomyocytes assessing its contribution in ROS production.

In the second part of the work we focused our attention on the role of MAO in phagocytic cells, as macrophages (MΦ) largely contribute to cardiac pathologies by ROS formation, cytokine release, tissue healing and cellular recruitment.

Although it is well-known that ROS play a key role in MΦ differentiation and activation, the sources of ROS involved in these processes are still unclear. Thus we hypothesized that MAO could be involved in the regulation of MΦ metabolism. To this aim MAO expression and its corresponding pathway of induction were investigated and characterized. Next, the contribution of MAO in ROS formation was assessed during differentiation and polarization processes. For this purpose, we assessed its contribution by means of ROS level measurements after treatment with differentiating stimuli (GM-CSF, MCSF) and also under polarization stimuli (LPS, IL-4 and IL-13). Cytofluorimetric analyses and ELISA assays were performed to assess the phenotypic MΦ differentiation to M1 or M2 subtype and these enabled to evaluate MAO inhibition effects on differentiation process.

7. RESULTS

7.1 The increased availability of MAO substrates upon oxidative stress induces MAO activation in perfused isolated hearts

It has been demonstrated that MAOs contribute to the oxidative stress occurring in hearts subjected to pathological conditions, such as cardiac reperfusion injury and decompensated hypertrophy, and that their inhibitors significantly protect the heart in these models. However, the molecular mechanisms underlying MAO activation are still unclear.
For this reason, in collaboration with the University of Bari, we quantitated and identified amines, potential MAO substrates, by MS analysis in mouse Langendorff perfused hearts subjected to two protocols of oxidative stress. We exploited the following procedures: (i) 1mM H_2O_2 perfusion for 15' and (ii) 40' ischemia followed by 15' reperfusion (IR).
The comparison of the amine contents, in the absence and presence of MAO inhibition, allowed the identification of the molecules that are potentially oxidized by this enzyme.

7.1.1 Oxidative stress protocol by H_2O_2 perfusion

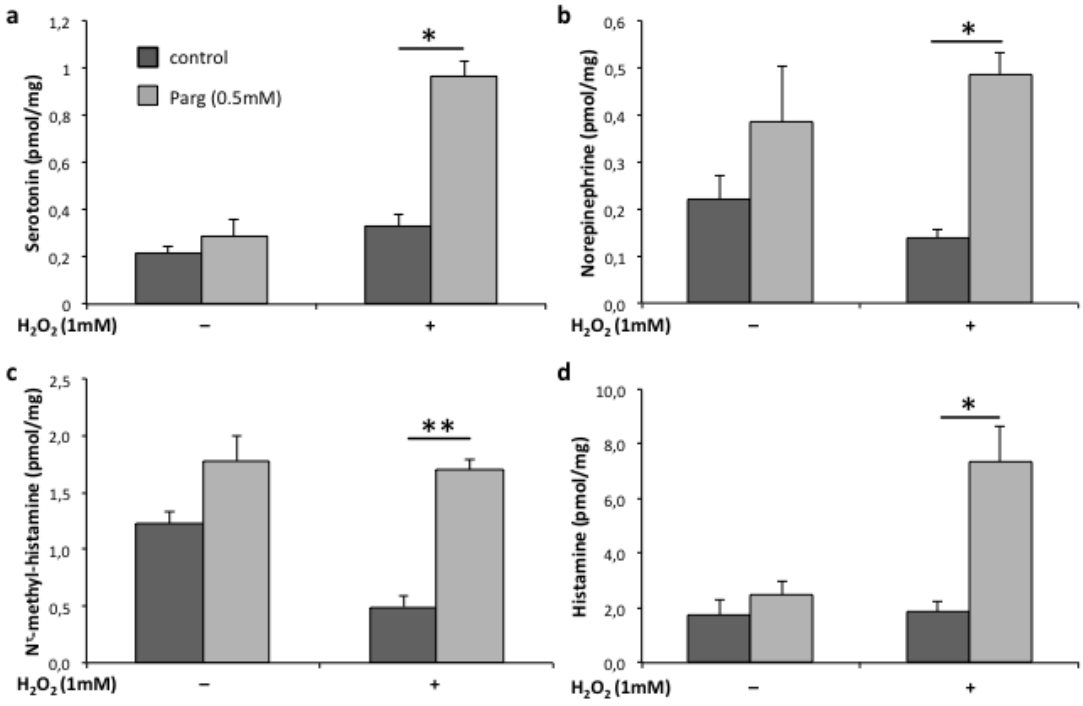

Figure 10. Identification and quantification of MAO substrates in hearts perfused with H_2O_2. Isolated mouse hearts were Langendorff-perfused in the absence and presence of pargyline (0.5mM) added 10' before the perfusion with a buffer containing H_2O_2 (1mM). In parallel, hearts were perfused in the absence of H_2O_2. They were frozen in liquid N_2 and analysed by MS. **p<0.05, **p<0.01*

The MAO inhibitor pargyline, added to the perfusion buffer 10' before the protocol of oxidative stress, caused a relevant increase in the heart content of typical MAO substrates: 5-HT, NE as well as N^{τ}-methylhistamine (NMH) and its precursor histamine (HA) (fig. 10). It is worth mentioning that NMH represented, together with its precursor, HA, the most abundant amine that accumulates in this experimental model of cardiac injury. Furthermore, the accumulation of MAO substrates upon pargyline exposure correlated with a reduced MAO-dependent production of H_2O_2, as detected by DHE staining in heart cryosections (fig. 11).

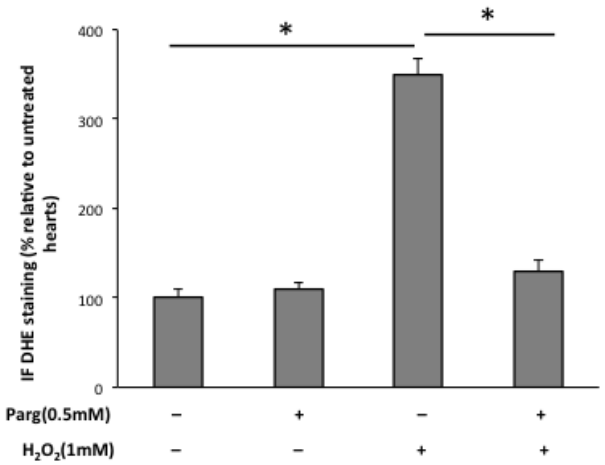

Figure 11. ROS levels upon H_2O_2 heart perfusion is decreased by MAO inhibition. Isolated mouse hearts were Langendorff-perfused in the absence and presence of pargyline (0.5mM) added 10' before the perfusion in the absence or in the presence of H_2O_2 (1mM). Heart slices were incubated 20' with DHE (5μM) and visualized by fluorescence microscopy. Fluorescence intensity was quantified using ImageJ software. **p<0.05 vs control*

The correlation between the decreased ROS formation in the presence of pargyline (fig. 11) and the corresponding accumulation of MAO substrates, suggests that H_2O_2 perfusion acts as a trigger for MAO activity increasing the availability of its substrates. Indeed, several studies indicate H_2O_2 as a key modulator and inducer of different metabolic pathway, supporting the idea that ROS could represent an important trigger also for MAO, in physiological and pathological conditions.

7.1.2 Oxidative stress protocol by IR

The increase in MAO substrates upon pargyline addition was observed also in hearts subjected to a second protocol of oxidative stress, i.e. post-ischemic reperfusion. In particular NMH (fig. 12a) and HA (fig. 12b) represented the most abundant amines accumulating in these hearts pre-treated with the MAO inhibitor, as in the protocol of H_2O_2 perfusion.
These results provide evidence for a role of HA metabolism in MAO activation.

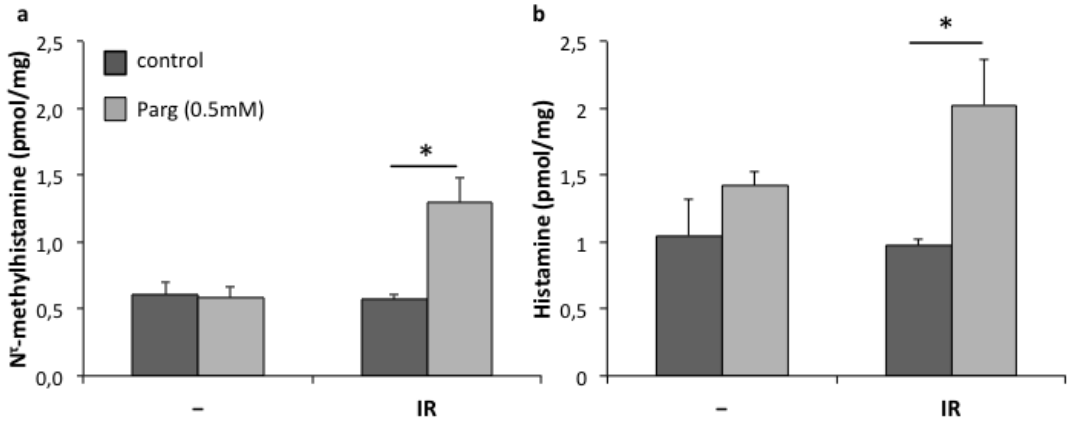

Figure 12. NMH and HA content in hearts perfused by IR protocol. Isolated mouse hearts were Langendorff-perfused in the absence and presence of pargyline (0.5mM) added 10' before the perfusion in aerobiosis or ischemia (40') followed by reperfusion (15'). a) NMH and b) HA were quantitated by MS. **p<0.05 vs control*

7.2 HDC is expresses in isolated cardiomyocytes

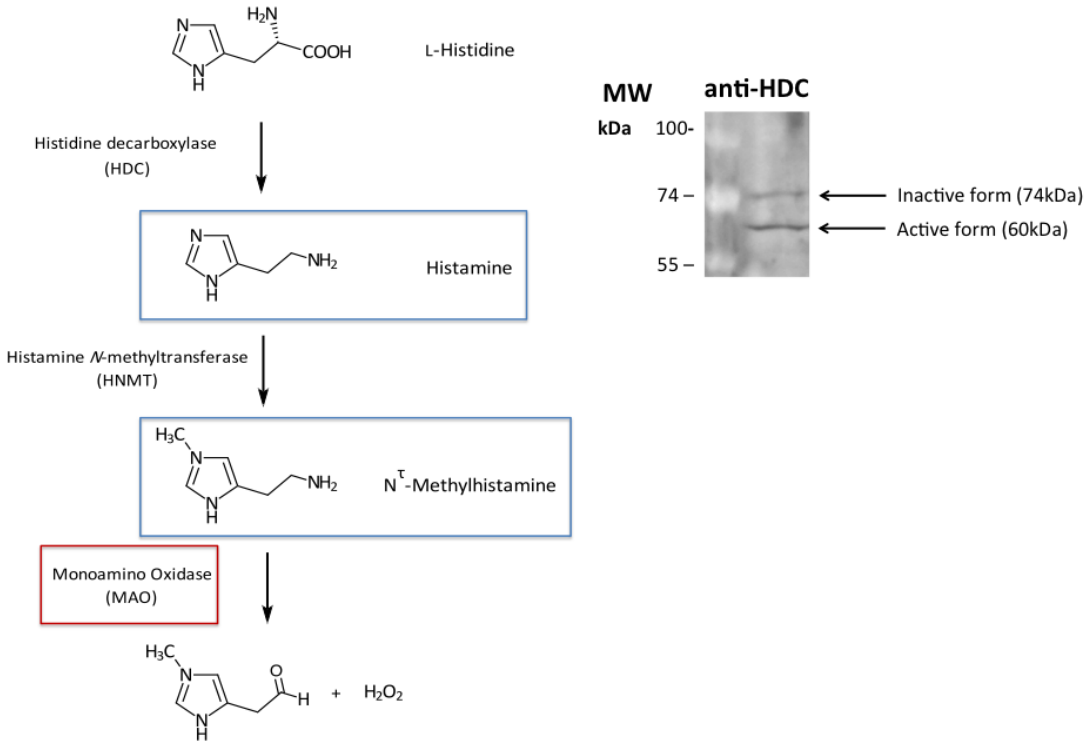

Figure 13. HDC expression in isolated cardiomyocytes HA is derived from histidine decarboxylation by L-histidine-decarboxylase (HDC) and then it is modified by histamine-N1-methyltransferase (HNMT) in its metabolite N$^{\tau}$-methylhistamine (NMH). Isolated NRVMs were lysed and separated by SDS/PAGE. Two bands, corresponding to the inactive form of the enzyme (74kDa) and to the active one (60kDa), were detected by western blot with anti-HDC antibody.

The scheme for HA metabolism (fig.13 left panel) reports the involved enzymes and, importantly, recalls that NMH is a MAO substrate, whereas HA is not, as reported in literature.[178, 179, 180]
It has already been verified that histidine-decarboxylase (HDC) and histamine-N-methyltransferase (HNMT) are expressed in heart homogenates.[181] In addition we provided evidence that also isolated cardiomyocytes are able to express HDC enzyme (fig. 13 right panel), providing HA themselves.

7.3 Cardiomyocyte as MAO substrate source

To evaluate whether isolated cardiomyocytes synthesize MAO substrates, potentially promoting MAO activity, we analysed isolated NRVM by MS.

metabolite	nmol/10^6cell
Histamine	0.0171
N^{τ}-methylhistamine	0.00066
Epinephrine	0.0011

Figure 14. Amine content in isolated neonatal cardiomyocytes. The identification and quantitation of the amines contained in NRVMs (5x10^^{6}cell/plate) were performed by MS analysis.

HA, NMH and epinephrine were found to be the most abundant amines in isolated cells (fig. 14), underlying a potential role for HA metabolism in the heart. Moreover, these data suggest the hypothesis that cardiac myocytes could promote MAO activity by synthesizing its specific substrates, i.e. NMH and epinephrine upon oxidative stress.

7.3.1 Oxidative stress *in vitro*

To test this hypothesis, the amine levels were quantitated in NRVMs exposed to H_2O_2 for 1h in the absence and presence of pargyline.

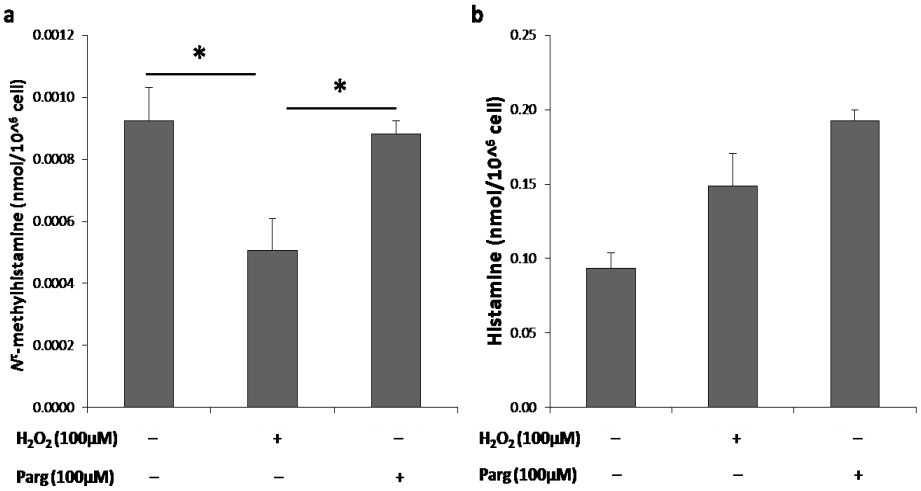

Figure 15. NMH and HA quantification in isolated NRVMs subjected to oxidative stress conditions. We quantitated by MS analysis a) NMH and b) HA content in NRVMs (5x10^6cell/plate) after 1h incubation with H_2O_2 (100μM) in the absence and presence of pargyline (Parg 100μM). **p<0.05*

MS analysis showed that, in conditions of oxidative stress, NMH accumulated upon MAO inhibition (fig. 15a), as in the *ex vivo* model, indicating H_2O_2 as a trigger for MAO activity. These results paralleled the decreased ROS levels due to pargyline, that was previously demonstrated in Prof. Di Lisa's laboratory (not shown).

7.4 Identification of MAO non-myocyte sources in the heart

To investigate the contribution of non-myocyte sources of MAO substrates under oxidative stress conditions we focused our attention on the synaptic terminals that innervate heart and commonly represent a pivotal source of neurotransmitters. For this purpose, mice were denervated by i.p. injection with 6-hydroxydopamine (6-OH-DOPA), a neurotoxic drug that deletes catecholaminergic neurons. After 24h hearts were excised and subjected to the IR protocol in the absence and presence of pargyline. To verify the efficacy of the denervation protocol, cardiac homogenates were tested by western blot for tyrosine hydroxylase (TH) (fig. 16), a functional marker for synaptic terminals.

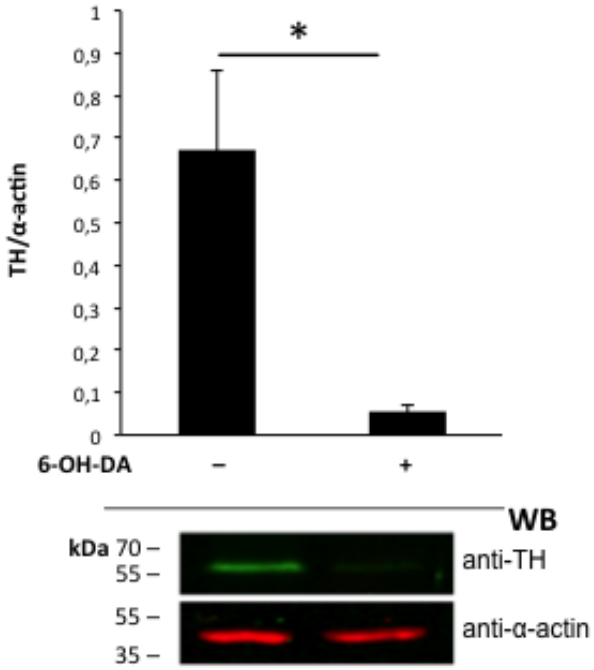

Figure 16. Denervation efficacy was assessed by tyrosine hydroxylase (TH) western blot. Mice injected with 6-OH-DOPA (0.1mg/g mouse weight) or with vehicle were sacrificed after 24h and subjected to aerobic perfusion or IR protocol. Denervation efficacy by 6-OH-DOPA (0.1mg/g mouse weight) was assessed by western blot analysis for TH antibody. Heart excised by denervated and vehicle-treated mice were homogenated and separated by SDS/PAGE. Band intensities were quantified by densitometry using ImageJ software and normalized to α-actin to check protein loading. **p<0.05 vs control*

A significant decrease of TH protein levels was observed after 24h of denervation (fig. 16).

7.4.1 Synaptic terminals significantly contribute to MAO substrate availability

Denervated and vehicle-treated hearts were then analyzed by MS to quantitate MAO substrates.

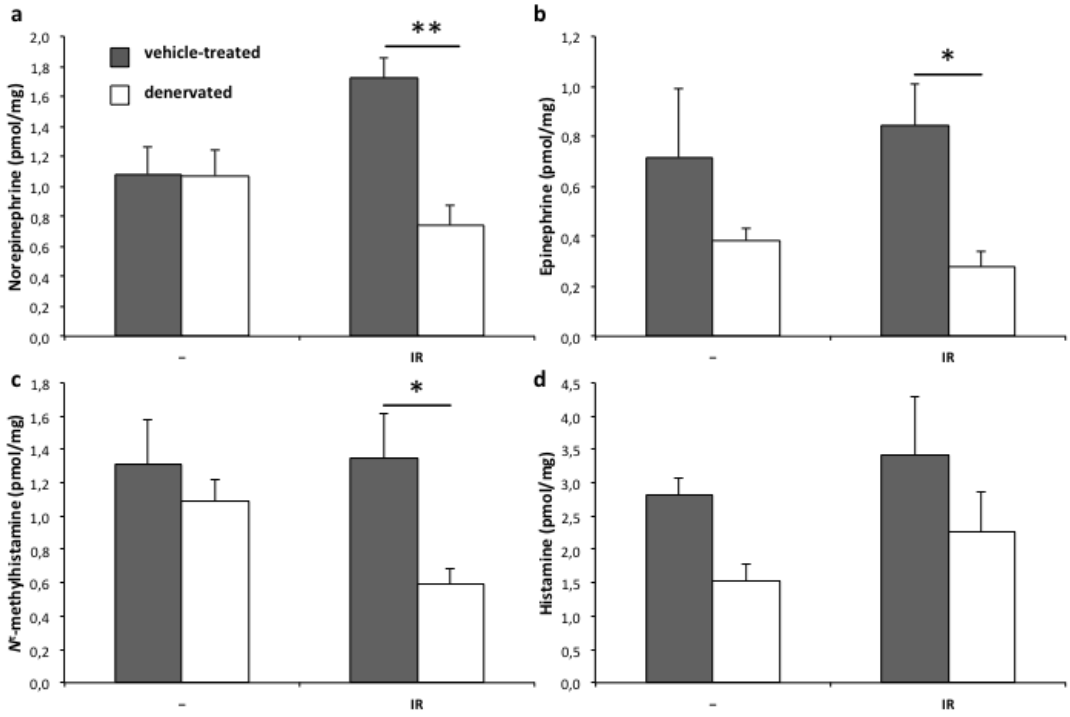

Figure 17. MAO substrate content in denervated hearts as compared to vehicle-treated controls. Mice injected with 6-OH-DOPA (0.1mg/g mouse weight) or with vehicle were sacrificed after 24h and subjected to aerobic perfusion or IR protocol. MS analyses were subsequently performed to identify and quantitate MAO substrates: a) NE, b) epinephrine, c) NMH, d) HA. **p<0.05, **p<0.01*

The analysis showed significant differences between denervated and vehicle-treated hearts. Upon IR treatment, denervated hearts displayed NE, epinephrine and NMH decrease as compared to vehicle-treated hearts (fig. 17a-b-c). However HA content was not significantly decreased by denervation. We considered that other HA sources in heart tissue, such as mast cells,[182] are not affected by chemical denervation, so they can balance the synaptic terminal contribution, justifying the mild decrease of HA upon denervation (fig. 17d).
Taken together, our findings showed that synaptic terminals represent one of the main non-myocyte sources for MAO substrates under oxidative stress conditions. Furthermore, LDH assay of the related effluents indicates a protective effect linked to denervation treatment (fig. 18), already discussed in other papers.[182, 184, 185] The protection correlated to the decreased substrate availability for MAO activity. Indeed, these data demonstrate that under pathological conditions a larger availability of substrates fuels MAO activity exacerbating ROS formation, which plays a crucial role in IR-induced injury.

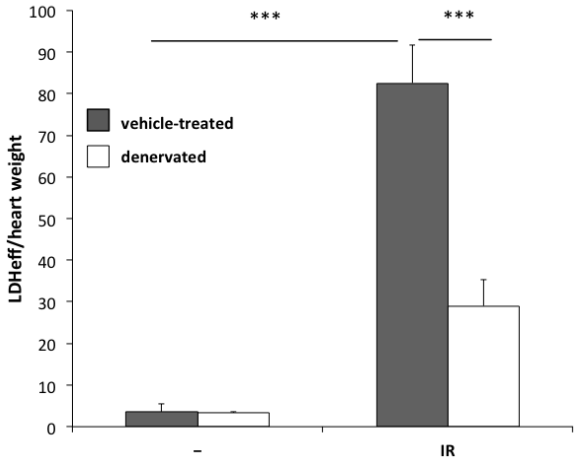

Figure 18. Denervated hearts are protected against IR-induced injury. Mice injected with 6-OH-DOPA (0.1mg/g mouse weight) or with vehicle were sacrificed after 24h and subjected to aerobic perfusion or IR protocol in the absence and presence of pargyline (0.5mM). Effluents collected during post-ischemic reperfusion (40'-15') were analyzed by LDH assay to quantitate lactate dehydrogenase release. ****p<0.001*

To better characterize the relevance of HA and NMH in injured hearts, we evaluated the contribution of other sources besides synaptic terminals. For this purpose we subjected denervated hearts to IR protocol in the presence and absence of pargyline.

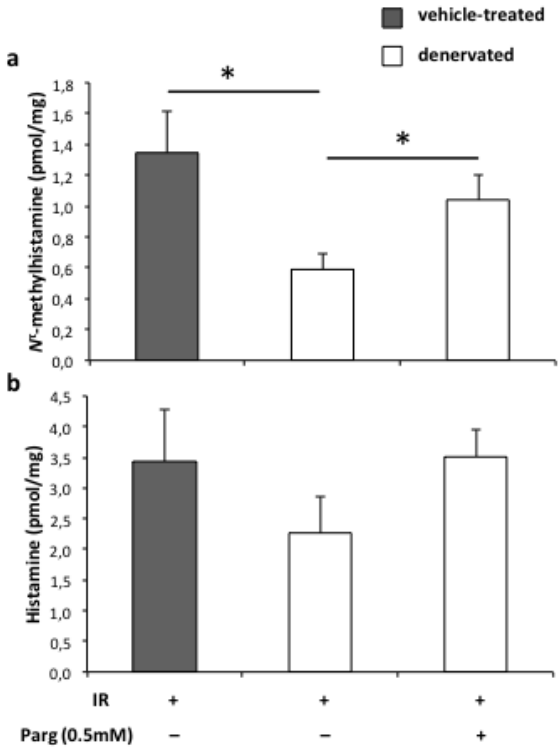

Figure 19. NMH and HA content in denervated hearts in comparison to vehicle-treated controls upon MAO inhibition. Mice injected with 6-OH-DOPA (0.1mg/g mouse weight) or with vehicle were sacrificed after 24h and subjected to ischemia (40') followed by reperfusion (15') in the absence and presence of pargyline (0.5mM). MS analysis were subsequently performed to quantitate a) NMH and b) HA. **p<0.05*

MS analyses suggest that, although synaptic terminals give a relevant contribution to NMH availability (fig. 17c), other sources promote MAO activity as demonstrated by the corresponding accumulation upon MAO inhibition (fig. 19a). However, its extent of accumulation is lower as compared to non denervated hearts. The other sources can be represented by immune cells () and also by cardiomyocytes themselves, as reported (fig. 14).

7.5 HA contribution to induce oxidative stress *in vitro* through MAO activation

To elucidate the mechanism underlying the effect of HA and its metabolite in determining oxidative stress, NRVMs were exposed to HA in the absence or presence of pargyline.

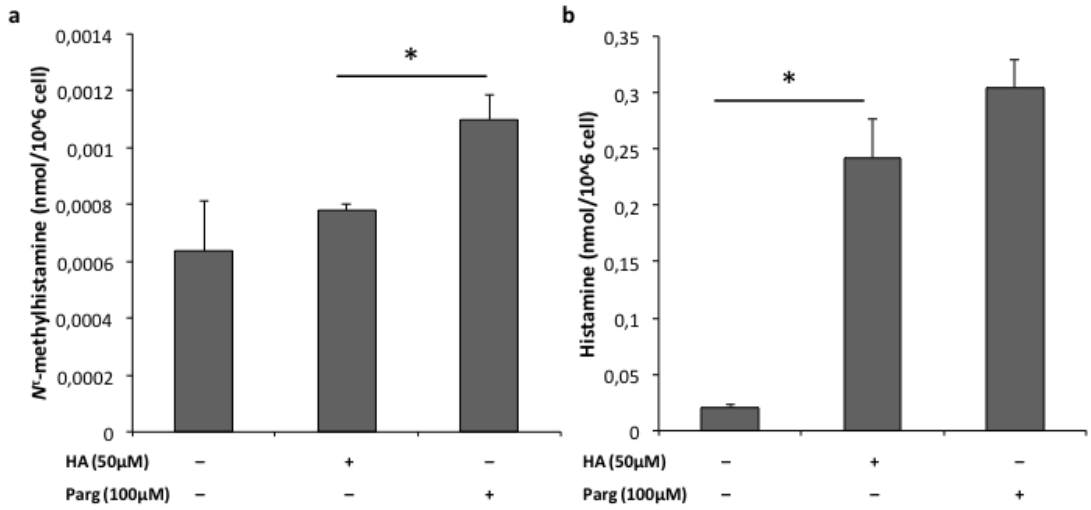

Figure 20. NMH and HA content in isolated NRVMs upon HA exposure in the presence or absence of MAO inhibition. By MS analysis a) NMH and b) HA content was quantitated in NRVMs (5x10^6cell/plate) incubated 1h with HA (50µM) in the absence and presence of pargyline (Parg 100µM). **p<0.05*

HA addition to NRVMs caused its own intracellular increase (fig 20b). This indicates that HA is transported, through the OCT3 amine transporter into the cardiomyocytes.[186, 187] Importantly, the increased levels of HA are accompanied by NMH formation, that becomes evident in the presence of pargyline.
In parallel we also performed ROS measurements of NRVMs incubated with HA.

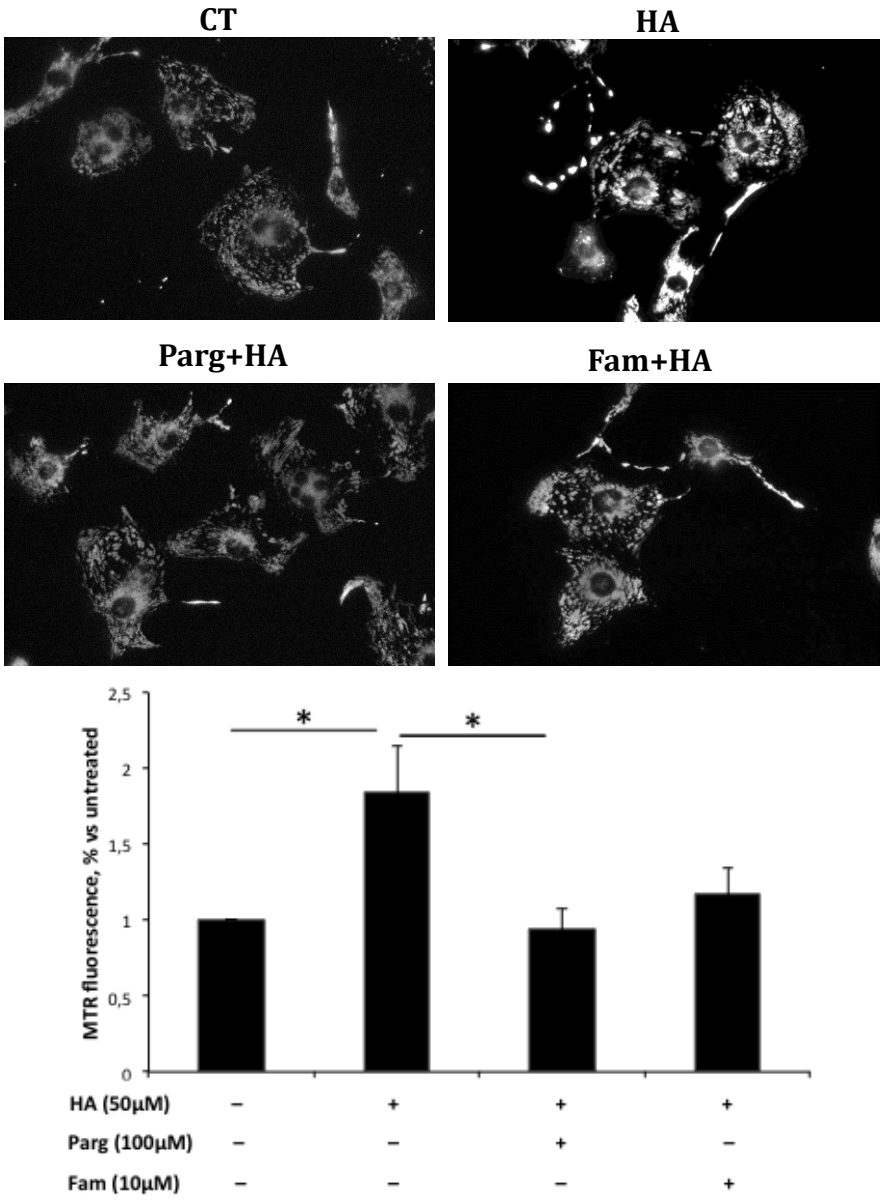

Figure 21. ROS level measurement in NRVMs after HA exposure. NRVMs were treated for 1h with HA (50µM) in the absence and presence of pargyline (100µM) or famotidine (10µM). Inhibitors were added 30' before the treatment. ROS levels were visualized by means of MitoTracker® Red CM-H2Xros (MTR)(20nM) fluorescence using a 63X objective. **p<0.05*

HA induced an increased ROS formation that was reduced in the presence of pargyline (fig. 21). Taken together, these data support the hypothesis that NMH availability increases due to the activation of HA metabolism, i.e. HA enters into cardiomyocytes and is metabolized to NMH that, in turn, fuels MAO activity.
We also considered that HA activates its specific receptor 2, expressed on the plasma membrane of cardiomyocytes, to increase vascular permeability and induce apoptosis.[188, 189] Therefore, to verify the potential contribution of the histamine-2-receptor (H2R) pathway in the increase of ROS levels, its activation was tested by means of a specific inhibitor, famotidine.[190, 189] Famotidine did not significantly reduce the HA-dependent ROS level induction (fig. 21), providing evidence that the H2 receptor-dependent mechanism does not play a major role in the MAO-dependent oxidative stress induced by histamine.

7.6 MAO in human Monocytes/Macrophages

It has been largely shown that inflammation is usually involved in cardiac diseases and cells involved in inflammatory response such as mast cells, monocytes, macrophages (MΦ), might release substrates for MAO activity and consequently increase the oxidative stress. Furthermore, MAO itself may contribute to ROS production inside immune phagocytic cells, on which we focused our attention.
As already described in the Introduction, the role of MAO in phagocytic cells has not been clearly defined.[105] Thus, to better characterize MAO role in phagocytic cells, we investigated MAO-dependent H_2O_2 involvement in MΦ development and activity.
Firstly, MAO protein levels were measured in human monocytes (Mn) and MΦ, M1 and M2, by Western blot.

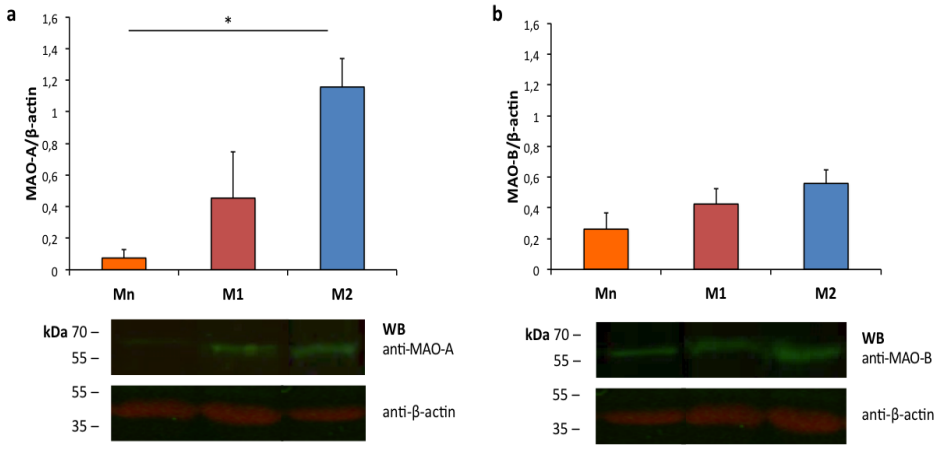

Figure 22. MAO-A and –B expression levels in human Mn in comparison to derived MΦ. Mn were isolated from buffy coat as previously described in *Material and Meth*ods. 200x10^6 cells were lysed immediately after isolation (Mn). 2x10^6 Mn perwell were plated and differentiated in MΦ, M1 and M2, for seven days in presence of the specific stimuli, GM-CSF (100ng/ml) and M-CSF (100ng/ml) respectively. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin to check protein loading. **p<0.05 vs Mn*

Both MAO-A and –B isoforms were expressed in M1 and M2 differentiated MΦ, at difference of their precursor Mn that displayed low levels of the enzyme. M1 MΦ expressed lower MAO protein levels than the M2 population, and the A isoform showed significant differences (fig. 22a) whereas the isoform B did not show significant increases (fig. 22b).
Based upon the literature we polarized cells in different ways to evaluate MAO-A expression: we compared IL-4 and IL-13 combinatorial treatment (IL-4+IL-13), which are anti-inflammatory stimuli, to LPS exposure, that is a pro-inflammatory one.

7.6.1 Pro-inflammatory and anti-inflammatory stimulus induction of MAO-A expression in human M2 MΦ

Firstly, we polarized differentiated MΦ with LPS and assessed the expression of MAO-A, the most relevant isoform as previously demonstrated (fig. 23).
LPS is a common stimulus used to simulate an inflammatory state that polarizes MΦ through TLR4 activation.[191]

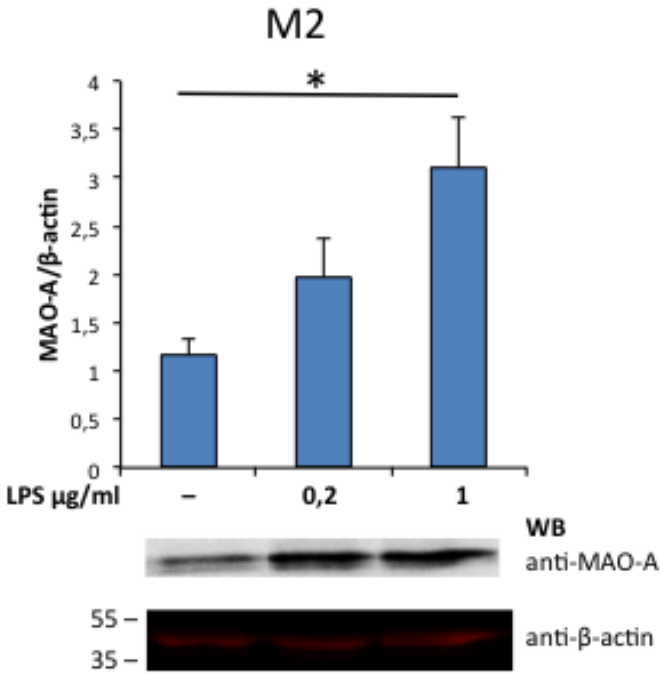

Figure 23. MAO-A expression levels in polarized MΦ M2. M2 MΦ were incubated with different doses of LPS (0.2, 1µg/ml) for 24h. After LPS exposure cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin to check protein loading. **p<0.05 vs control*

By western blot analysis we demonstrated that LPS induces MAO-A expression in a dose-dependent manner in M2 MΦ (fig. 23).
These data support and validate recent studies that propose MAO-A as a possible marker for M2 MΦ.[106, 107, 109] Therefore, we compared LPS treatment to IL-4+IL-13 exposure in M2 MΦ, to assess the protein level induction linked to opposite stimuli.

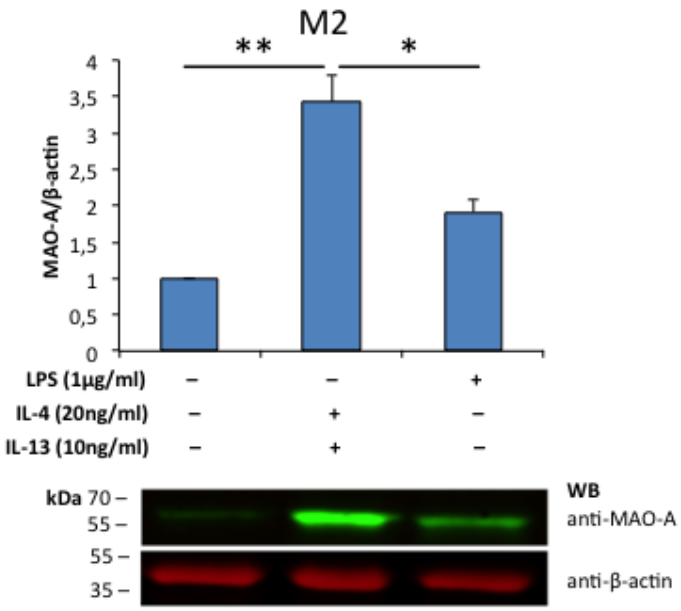

Figure 24. MAO-A expression levels in polarized M2 MΦ upon pro- and anti-inflammatory stimuli. Cells differentiated in M2 MΦ were incubated for 24h with LPS (1µg/ml) or with IL-4 (20ng/ml) and IL-13 (10ng/ml). At the end of the treatment cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin to check protein loading. **p<0.05, **p<0.01*

IL-4 and IL-13 combination induced higher protein levels as compared to LPS exposure. This result indicates that both stimuli (i) LPS and (ii) interleukin combination represent MAO inducers, even if they are commonly thought to promote opposite polarizations, pro- and anti-inflammatory respectively.

7.6.2 MAO-A induction pathway in human M2 MΦ

LPS and IL-4+IL-13 treatments have been reported to induce a MAPK cascade through the activation of their corresponding receptors. Among these kinases p38 MAPK has been shown to be involved in Sp1 activation in phagocytic cells.[192, 193, 194] Furthermore, as reported in the Introduction, this transcription factor induces MAO expression.[123, 124, 125, 126] Therefore, we hypothesized a possible MAO-A induction pathway that involves these two factors: p38MAPK and Sp1 factor. To verify this hypothesis, two specific inhibitors were used: SB202190, a p38 inhibitor, and mithramycin, a specific Sp1 inhibitor. M2 MΦ were exposed to both the activation treatments (LPS and IL-4+IL13) in the absence and presence of the two corresponding inhibitors.

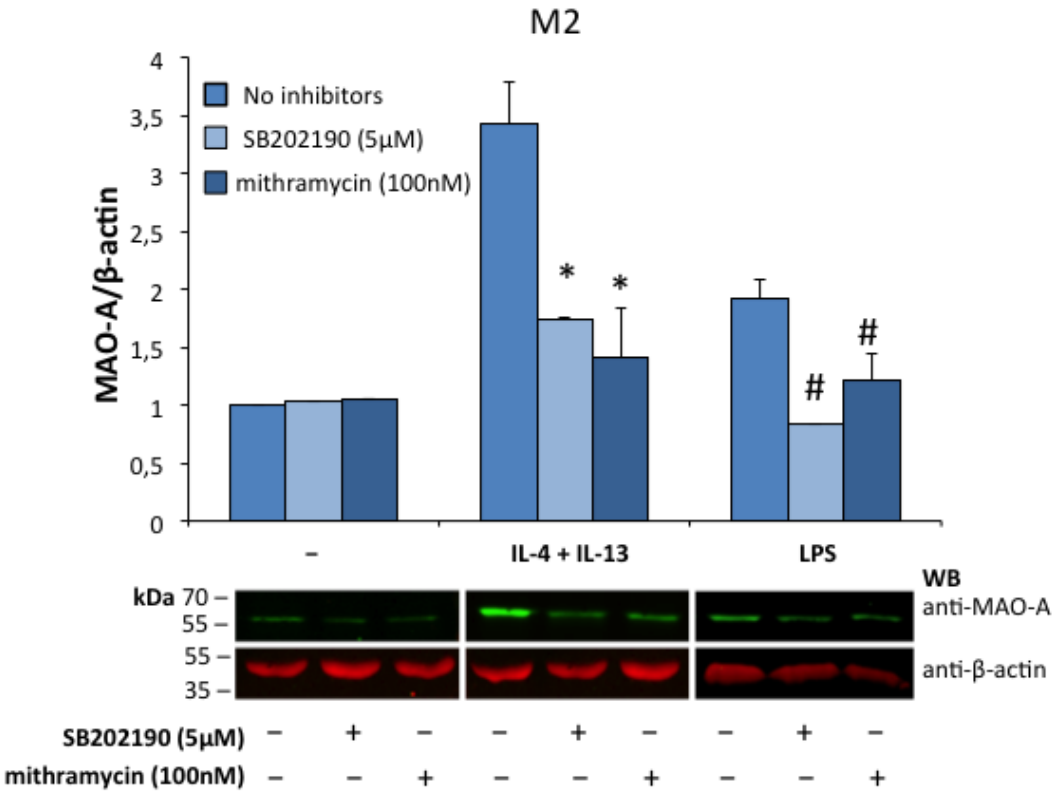

Figure 25. MAO-A expression levels in polarized M2 MΦ upon pro- and anti-inflammatory stimuli in the presence and absence of MAPK p38 inhibitor and Sp1 factor inhibitor. Cells differentiated in M2 MΦ were pre-treated for 30' with SB202190 (5μM) or mithramycin (100nM) and then incubated 24h with LPS (1μg/ml) or IL-4 (20ng/ml) and IL-13 (10ng/ml). At the end of the treatment cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin to check protein loading. **#p<0.05*

Western blot proved that p38 and Sp1 inhibitors prevented MAO expression upon both the treatments, LPS and IL4+IL-13 (fig. 25), validating our hypothetical induction mechanism.

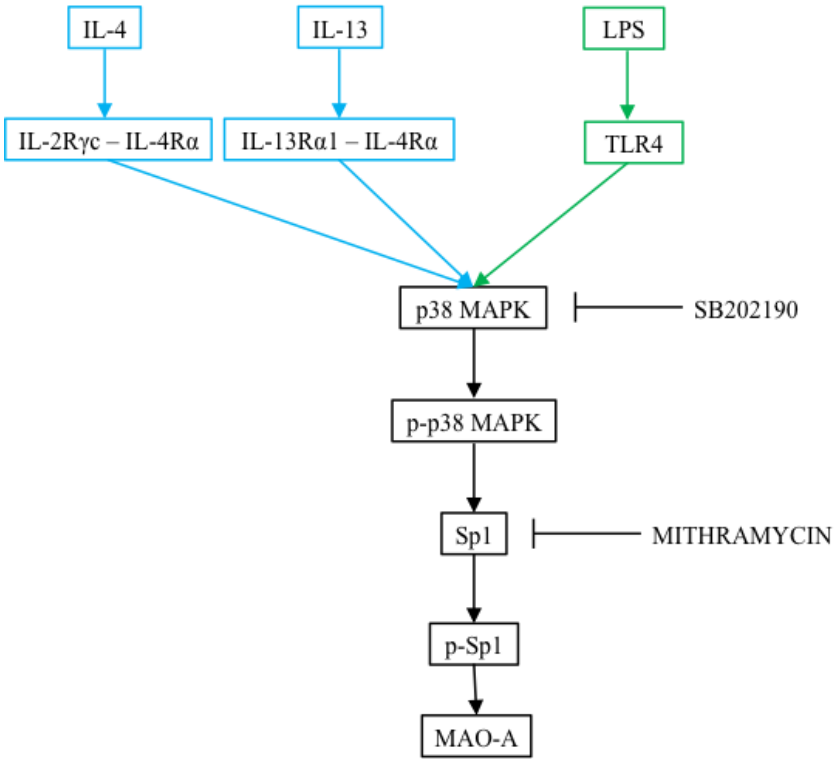

Figure 26. Scheme of MAO induction pathway in MΦ M2 upon LPS and IL-4+IL-13 treatments.

These latter results describe a new pathway of MAO-A induction in M2 MΦ (fig. 26). Moreover, these data suggest that MAO may be involved in physiopathological MΦ processes.

7.6.3 MAO role in human MΦ differentiation

It is known from the literature that ROS are involved in MΦ differentiation.[89, 92] Therefore, we speculated a possible role for MAO-A-dependent ROS production during cell development process. We measured MAO-A expression during the differentiation period of 7 days.

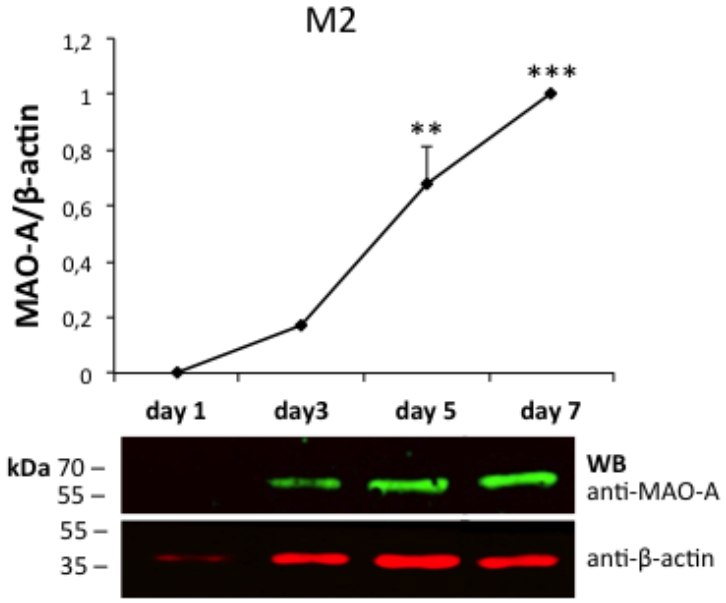

Figure 27. Time-dependent expression of MAO-A along M2 MΦ differentiation. Cells were incubated in presence of M-CSF (100ng/ml) and every two days were collected to verify MAO-A expression levels. At the end of the treatment cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin to check protein loading. ***p<0.01, ***p<0.001 vs day 1*

These experiments showed that MAO-A induction is time-dependent (fig. 27) and that it reaches appreciable expression levels since the third day of differentiation.

As detailed in the Introduction (4.6.4), ROS are key regulators of MΦ differentiation. Thus, to understand if MAO could contribute to phagocytic cell development by its H_2O_2 production, we measured ROS levels in both MΦ M1 and M2 at the seventh day of differentiation.

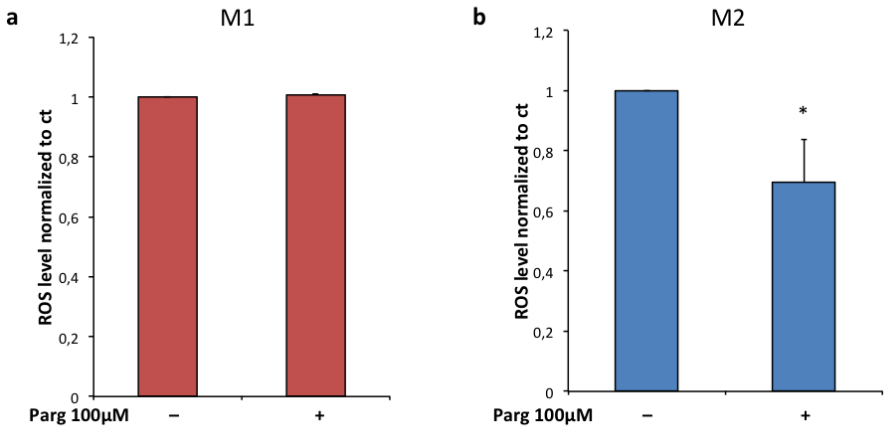

Figure 28. MAO-dependent H_2O_2 contributes to redox homeostasis of differentiated M2 MΦ. Cells were treated during the differentiation period in the presence and absence of pargyline (100μM). At the seventh day they were incubated for 30' with DCFDA (10μM) before the cytofluorimetric analysis. Dying cells were excluded by the use of Propidium Iodide, as described in Materials and Methods (5.8.1). **p<0.05*

Pargyline reduced ROS levels only in M-CSF-differentiated M2 MΦ, and not in GM-CSF differentiated M1 (fig. 28b). These results indicate that MAO-dependent H_2O_2 contributes to ROS homeostasis of differentiated M2 MΦ, suggesting a role for MAO in the development of M2 phenotype. To verify this hypothesis we tested the expression of two specific markers, CD80 and CD163, corresponding to the M1 and M2 phenotype, respectively.[195, 196] These two proteins are expressed on MΦ plasma membrane and they represent targets to distinguish the two MΦ populations.

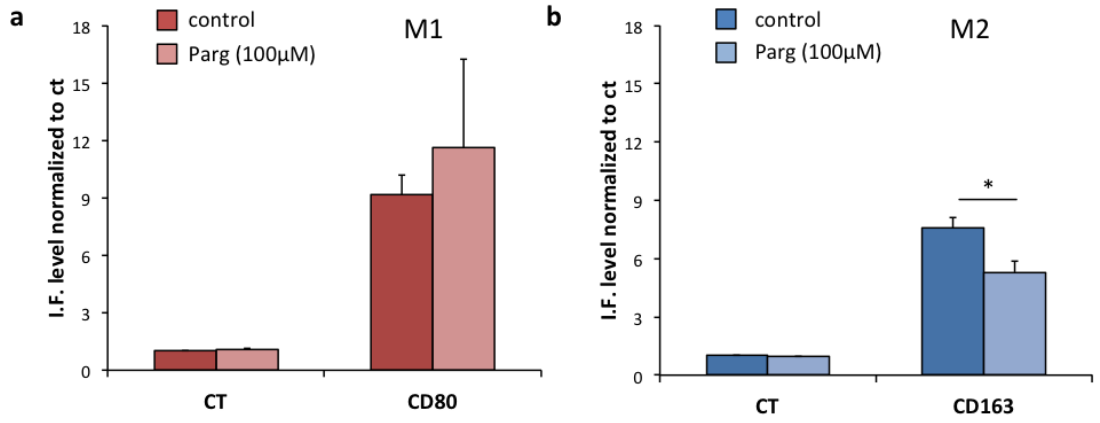

Figure 29. MΦ markers expression in the presence and absence of pargyline. After seven days of differentiation in the presence or absence of pargyline (100μM) M1 and M2 MΦ either have been tested using specific FITC-conjugated antibody for a) CD80 (M1) and b) CD163 (M2). Cells were detached and incubated for 30' in ice with the corresponding antibodies. Samples were analysed by cytofluorimetry. Dying cells were excluded by the use of Propidium Iodide, as described in Materials and Methods (5.8.2). **p<0.05*

MAO inhibition decreased CD163 expression (fig. 29b), while it did not affect CD80 (fig. 29a), supporting data that linked MAO activity to M2 phenotype.

Therefore, ROS level decrease (fig. 28b) and CD163 reduced expression (fig. 29b) suggest that MAO activity contributes to M2 MΦ development.
To define that MAO-dependent ROS production is involved in M2 MΦ differentiation, we considered a possible interaction with one of the most common MAPK involved in cell development and homeostasis, ERK.
M-CSF has been reported to induce ROS production through its specific receptor [197] that leads to a MAPK cascade involving ERK activation.[89, 198, 199] However, the mechanism that induces ROS formation and activates this signalling remains unclear. Thus we evaluated ERK phosphorylation in the absence and presence of pargyline during differentiation.
Based on MAO-A expression levels along M2 MΦ differentiation (fig. 27), we considered day 5 and 7.

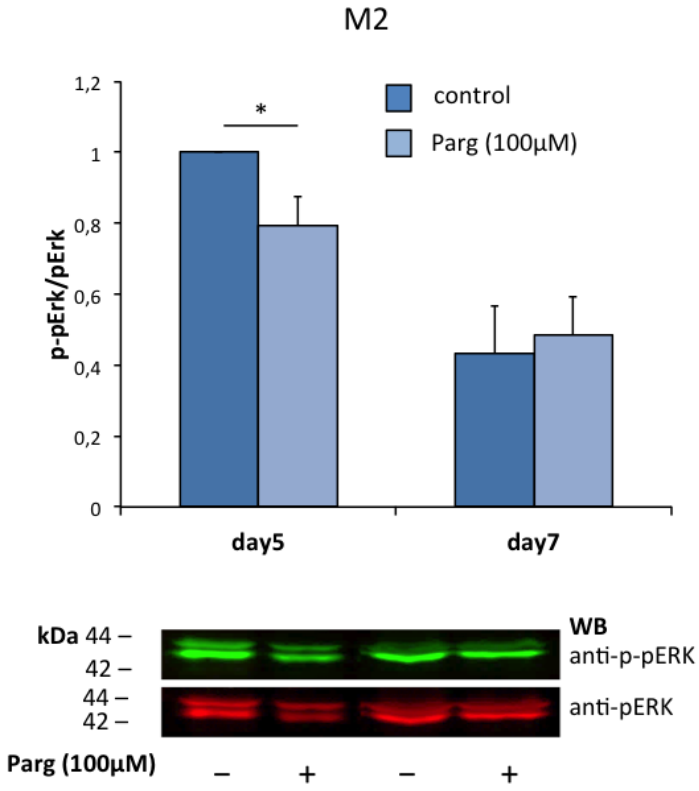

Figure 30. ERK expression and activation during M2 MΦ differentiation. Cells were treated during the period of differentiation in the presence and absence of pargyline (100µM). M2 MΦ were collected at the fifth and seventh day. Cell lysates were separated by SDS/PAGE and tested by western blot for ERK antibodies (anti-Phospho-p42/44(ERK) / anti-p42/44(ERK)). Band intensities were quantified by densitometry using ImageJ software. **p<0.05*

M2 MΦ at the seventh day did not show any difference between cells treated or untreated with MAO inhibitor, instead at the fifth day of differentiation a significant decrease in ERK phosphorylation occurred (fig. 30). These data provide evidence the involvement of MAO activity in MΦ M2 differentiation and its implication in ROS-dependent ERK MAPK activation.[89] At the end of M2 MΦ differentiation (day 7) pargyline did not display any effect on ERK phosphorylation. This indicates that the maximal activation of ERK activity occurs at the fifth day of differentiation, when cells are exposed to M-CSF. Indeed, as described in Material and Methods (5.1.2), at the fifth day of

differentiation half part of the medium was changed. Therefore, new M-CSF addition to the cells led to ROS-dependent ERK MAPK activation.[89]

7.6.4 MAO role in human MΦ polarization

Given the increased MAO expression previously shown upon MΦ activation by LPS (fig. 25), we hypothesized that MAO-dependent H_2O_2 could contribute to MΦ ROS production both for bactericidal activity and MΦ homeostasis. To this aim M1 and M2 MΦ were treated with LPS for increasing time and the corresponding ROS levels were measured.

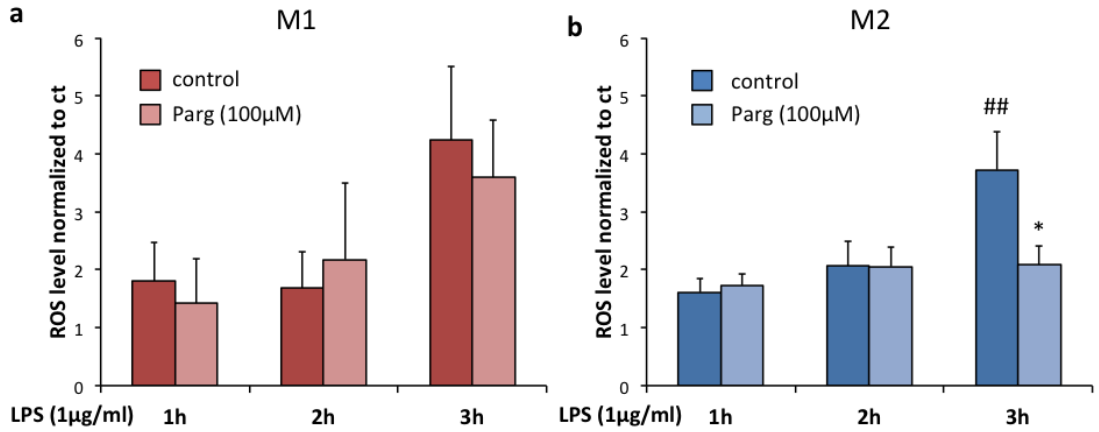

Figure 31. MAO contributes to M2 MΦ polarization by its H_2O_2 production upon LPS exposure. Cells were treated for increasing time with LPS (1μg/ml) in the presence and absence of pargyline (100μM). They were incubated for 30' with DCFDA (10μM) before the cytofluorimetric analysis. Dying cells were excluded by the use of Propidium Iodide, as described in Materials and Methods (5.8.1). **p<0.05 vs 3h parg-LPS, ##p<0.01 vs 1h LPS*

In the absence of LPS M1 pro-inflammatory MΦ produced high levels of ROS as compared to M2 MΦ (values not reported), however only M2 MΦ showed a significant increase of ROS levels upon LPS treatment and a relevant reduction at 3h upon pargyline exposure (fig. 31b).
Based upon a recent study of Bhattacharjee and coworkers,[109] we investigated also if IL-4+IL-13 treatment could induce MAO-dependent ROS formation, supported by the addition of a typical MAO substrate, tyramine. However, no significant ROS increase was measured in our experiments in opposite to Battacharjee (data not shown). Probably this disagreement is due to the different protocol of cell differentiation and polarization that we used.[109]
Given the ROS decrease at 3h LPS treatment in presence of pargyline (fig. 33b), we tested a possible correlation between this effect and M2 MΦ polarization process. To this aim we performed ELISA assay for two specific cytokines IL-10 and IL-6.

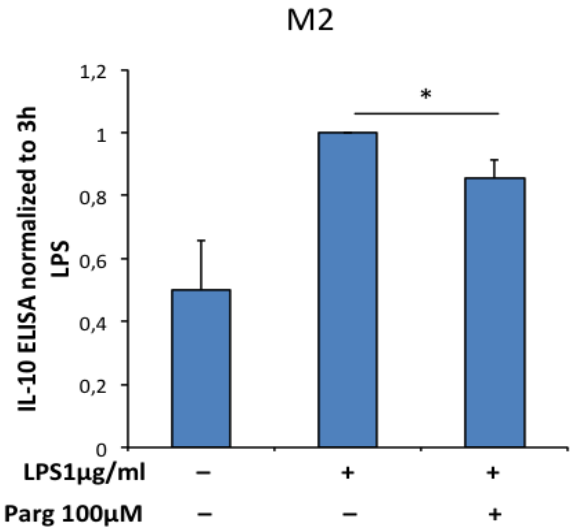

Figure 32. IL-10 in MΦ M2 under LPS treatment. 2x10^6 M2 MΦ perwell were polarized in the presence and absence of LPS (1µg/ml) in the absence and presence of pargyline (100µM). After 3h, supernatants were collected and IL-10 was quantitated by ELISA assay. **p<0.05*

IL-10 represents an anti-inflammatory cytokine released by MΦ M2, which was reduced upon MAO inhibition as shown (fig. 32). IL-10 is generally considered an anti-inflammatory cytokine, because it blocks the production of pro-inflammatory cytokines and the capacity of myeloid cells to efficiently activate T-cells.[200, 201, 202, 203]

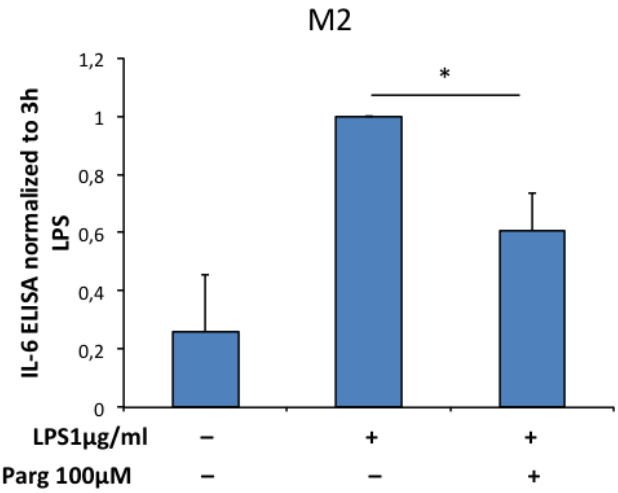

Figure 33. IL-6 in M2 MΦ under LPS treatment. 2x10^6 M2 MΦ per well were polarized in the presence and absence of LPS (1µg/ml) in the absence and presence of pargyline (100µM). After 3h, surnatants were collected and IL-6 was quantitated by ELISA assay. **p<0.05*

Also IL-6 release was decreased upon MAO inhibition (fig. 33). Although this cytokine is classically considered as a pro-inflammatory one, several papers has demonstrated its anti-inflammatory activity.[204, 205] IL-6 can promote regeneration and protection during infection and inflammation and on the other hand, this interleukin is needed for the activation of the immune system promoting mononuclear cell recruitment and T-cell apoptosis inhibition.[204]

Taken together, these findings (fig. 32-33) indicate that MAO is involved in M2 MΦ polarization, according to the ROS measurements obtained in the same conditions (fig. 31b).

7.7 MAO in murine models

In parallel to human MΦ we evaluated RAW264.7 immortalized murine cell line and BMDM primary culture. Our aim was to compare MAO activity and roles between the two organisms, human and mouse, to characterize the differences between the models. Moreover, we wanted to optimize the condition to study MAO role in phagocytic cells, due to the high variability of primary cultures.

7.7.1 MAO expression in RAW 264.7

By western blot analysis we assessed MAO expression after 24h of LPS exposure.

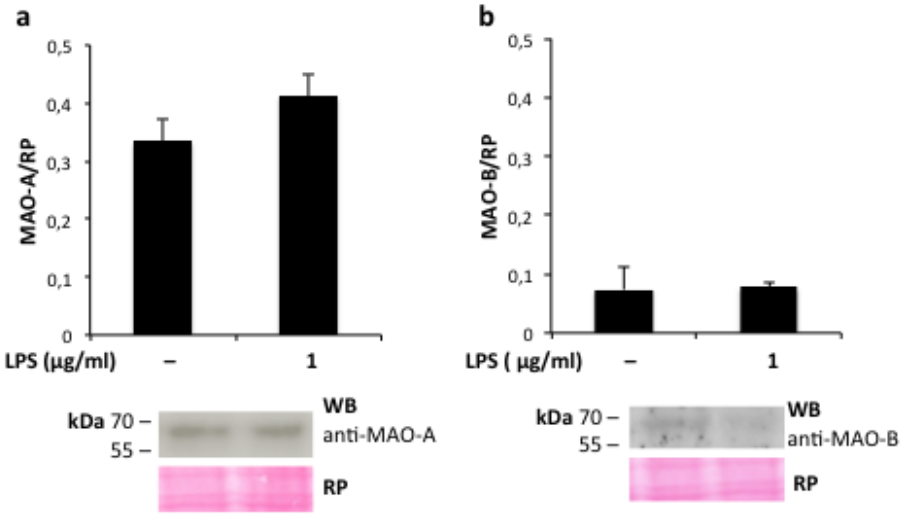

Figure 34. MAO-A and –B expression levels in murine MΦ cell line (RAW264.7) exposed to LPS. Cells were plated for 24h in the absence and presence of LPS (1µg/ml). At the end of the treatment cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to red ponceau to check protein loading.

Both the isoforms are expressed in RAW cells, however MAO levels did not increase upon LPS treatment (fig. 34), indicating a different responsiveness between primary human culture and immortalized murine cells.

7.7.2 MAO-A expression in M2 murine BMDM.

We assessed MAO-A expression levels at 24h and 48h in BMDM.

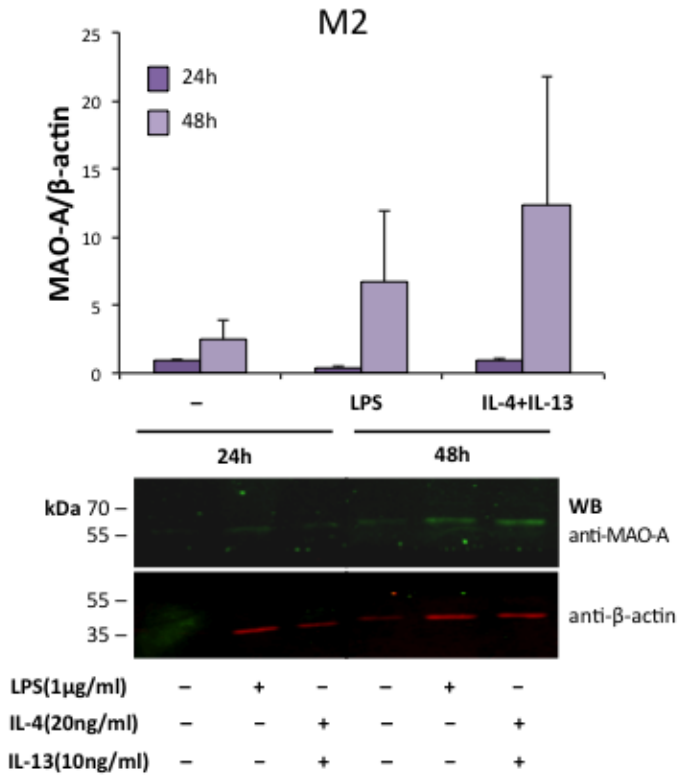

Figure 35. MAO-A and –B expression levels in murine M2 BMDM exposed to LPS treatment. Cells were plated for 24h and 48h in the absence and presence of LPS (1μg/ml). At the end of the treatments cell lysates were separated by SDS/PAGE and tested by western blot for MAO-A antibody. Band intensities were quantified by densitometry using ImageJ software and normalized to β-actin protein loading.

BMDM respond to LPS and IL-4+IL-13 treatment in a similar way to human MΦ but with a different kinetics, i.e. after 48h of exposure (fig. 35). These data suggest that MAO may act in a similar way both in human and murine primary culture.

7.7.3 MAO role in murine M2 BMDM differentiation

In order to characterize the role of MAO to BMDM differentiation, we performed ROS measurements at the end of the differentiation process in the presence and absence of pargyline.

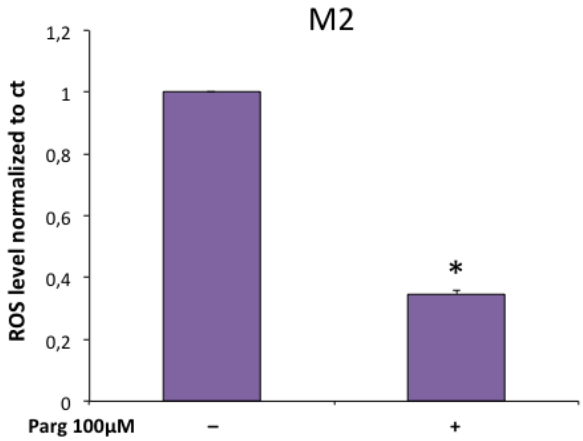

Figure 36. ROS levels in differentiated BMDM M2 MΦ at 7 days. Cells were differentiated with murine M-CSF (100ng/ml) specific stimulus, in the presence and absence of pargyline (100µM). At the seventh day cells were incubated for 30' with DCFDA (10µM) probe, before cytofluorimetric analysis. Dying cells were excluded by the use of Propidium Iodide, as described in material and methods chapter. **p<0.05*

M2 BMDM, as observed in human M2 MΦ, showed a decrease of ROS level reduction in the presence of pargyline (fig. 36), supporting a role for MAO also in mouse model in BMDM differentiation.

7.7.4 MAO role in murine M2 BMDM polarization

To test MAO involvement in M2 polarization, BMDM were exposed to LPS treatment for three hours at the seventh day as human samples. Furthermore, we performed the same experiment in parallel with the 48h treatment to assess MAO expression (fig. 35). At the end of these treatments, ROS measurements were performed by cytofluorimetric analysis.

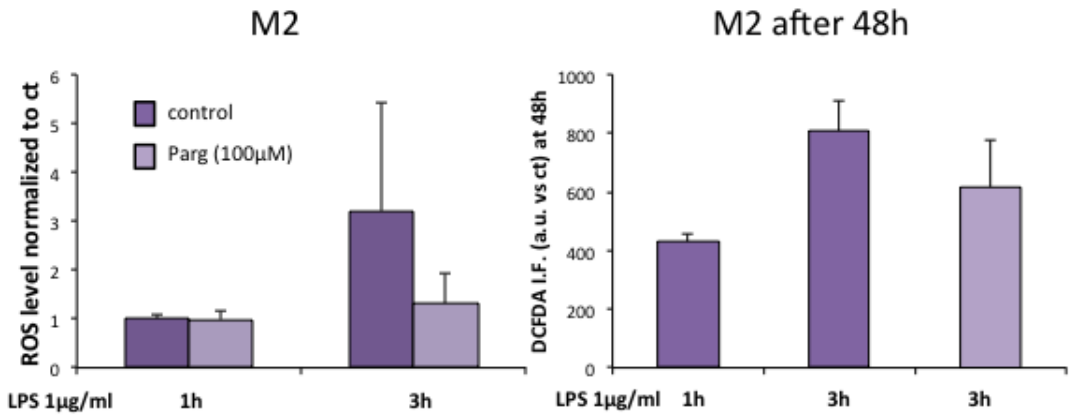

Figure 37. ROS levels in M2 BMDM in the presence and absence of MAO inhibition at different time point. Cells were treated for increasing time with LPS (1μg/ml) in the absence and presence of pargyline (100μM) and incubated for 30' with DCFDA (10μM) before cytofluorimetric analysis. This kind of experiment was performed at the seventh and the eighth day of BMDM differentiation. Dying cells were excluded by the use of Propidium Iodide, as described in material and methods chapter.

By these preliminary results it is likely to conclude that BMDM could respond as human MΦ, even if they report different time reactivity. Indeed, BMDM upon LPS exposure reached a peak at three hours that is blunted by pargyline (fig. 37a-b). However, it is necessary to deepen these studies to better characterize the differences between the phagocytic cells of the two organisms (human and mouse) and the MAO-related role.

8. CONCLUSIONS

The present study provides novel evidence about the regulation of MAO activity upon conditions of oxidative stress in the heart.

Here we proved for the first time that the increased availability of substrates plays a pivotal role in promoting MAO-dependent ROS production. Indeed, MAO inhibition causes accumulation of its substrates, especially NMH, in hearts subjected to *ex vivo* protocols of oxidative stress (H_2O_2 and IR). In parallel, ROS measurements showed also that MAO inhibition prevent cardiac damage indicating its implication in cardiac oxidative stress, as previously demonstrated.[29, 119] Therefore, it is likely that under pathological conditions a larger availability of substrates fuels MAO activity, increasing the formation of H_2O_2 that might amplify an initial oxidative stress.

Importantly, these results highlight for the first time a relationship between MAO activity and HA metabolism, that we had further confirmed by *in vitro* study in isolated cardiomyocytes.

HA is a low molecular weight amine (111.15g/mol) that acts as a neurotransmitter. It mediates intercellular signals promoting different physiological processes through its specific four G protein-coupled receptors (H1, H2, H3 and H4).[206, 207] Previous investigations have shown that endogenous HA and HA-receptors play highly significant roles in many cardiovascular diseases, such as hypertension,[208, 209] artherosclerosis[210, 211] and chronic heart failure.[212, 213] Additionally, some reports found that cardiac HA and its receptors are important in the development of abnormal cardiac rhythms, especially those induced by post-ischemic reperfusion.[214, 215, 216] However, no studies report any relation between HA metabolism and MAO activity in cardiac diseases.

As described in the results, histidine-decarboxylase (HDC) synthesizes HA that is metabolized by histamine-N-methyltransferase (HNMT) to NMH. Both these enzymes are expressed in the whole heart. In particular, we showed for the first time that also isolated NRVMs are equipped of HDC and contain histamine and its catabolite.

We also identified and evaluated the contribution of myocyte as MAO substrate source *per se*. We assessed that NRVMs actively participate to the oxidative stress induced by H_2O_2, through an increased availability of their substrates which amplify MAO activity.

Then, incubating NRVMs with HA, we confirmed the relationship between MAO activity and HA metabolism. Indeed, HA entry in isolated cardiomyocytes promotes the induction of MAO activity after its transformation in NMH, that is a MAO substrate - at difference of histamine. Moreover, parallel ROS measurements demonstrated that HA treatment induced ROS increase, which was reduced upon MAO inhibition.

To investigate the possible contribution of HA receptor-dependent signalling, we assessed the role of H2R in ROS increase upon HA exposure. The treatment of cardiomyocytes with famotidine, a H2R specific inhibitor, showed that ROS induction is not due to H2R-related pathway, a receptor-independent mechanism.

Regarding non-myocyte sources, synaptic terminals deletion provided evidence of the pivotal role of catecholaminergic neurons in MAO substrate availability.

Indeed, chemical denervation significantly decreased NMH availability upon IR injury. Furthermore, parallel release of LDH showed a protective effect linked to denervation treatment, according to previous studies. This protection[182, 184, 185] is likely to be linked to the reduction in MAO substrate availability and the consequent decrease in MAO activity.
The significant increase of NMH upon MAO inhibition in denervated hearts subjected to IR protocol indicates that other sources besides synaptic terminals can contribute to MAO activity. These alternative sources can be represented by immune cells (mast cells, monocytes, macrophages, neutrophils) and/or by cardiomyocytes, as already suggested.
On the other hand HA content was not significantly decreased by the deletion of catecholaminergic neurons. This can be explained by considering that HA is stored also in other cells of the heart, such as mast cells, which are resident in the tissue[182] or cardiomyocytes and these non-neuronal cells are likely not to be affected by chemical denervation.
Taken together, these data indicate that MAO substrates represent key regulators of MAO activity, highlighting an unexpected relationship between HA metabolism and MAO during cardiac damage. Importantly, we demonstrated the relevant contribution of (i) synaptic terminals as the main non-myocyte source and (ii) isolated cardiomyocytes. These results better define the mechanism through which MAO contributes to ROS formation upon cardiac injury, not only focusing on the intracellular enzyme but considering the environment and the intercellular interactions. Furthermore, our data support the relevant efficacy of MAO inhibitors in preventing increase oxidative stress and apoptosis in cardiac disease. Major advantages are given by MAO defined molecular structure, specific substrates, and clinically available inhibitors.[148] Moreover, MAO inhibitors are specific and not toxic in comparison to most common drugs for heart disease as beta-blockers, statin and calcium channel blocker.
In the second part of this work we better characterized MAO expression and role in human MΦ. Starting from the comparison between the pro- and anti-inflammatory MΦ phenotype (M1 and M2 respectively) we showed that both the isoforms are expressed in M1 and M2 MΦ either. According to several papers, only isoform A displayed significant levels in M2 phenotype [106, 107, 108] so we focused our attention on this isoform in M2 MΦ. Next, we showed that also a pro-inflammatory stimulus as LPS, commonly used to M1 MΦ polarization, induces MAO-A expression in M2 MΦ. Therefore we compared LPS to IL-4+IL-13 treatment. By this comparison we uncovered a novel pathway of MAO induction. Both the inhibitors for p38MAPK and Sp1 transcription factors reduced MAO-A expression, providing new evidence that LPS and IL-4+IL-13 treatments converge to a common induction pathway, despite their contrasting nature. These observations highlight the plasticity of phagocytic cells to respond to different stimuli and underline the difficulty to strictly classify them in distinct subtypes, as recently suggested.[67]
The effect of MAO inhibition on M-CSF dependent ROS production and induced ERK activity, demonstrated the involvement of MAO in the differentiation process of M2 MΦ. Moreover, we observed that MAO inhibition reduces CD163 expression in M2 MΦ, indicating that pargyline inhibits the correct M2 phenotype differentiation.

We also provided evidence of MAO contribution to ROS formation upon LPS treatment, suggesting that MAO is also involved in M2 MΦ polarization, whereas it is not in M1 MΦ polarization. ELISA assays for IL-10 and IL-6 supported the previous observations. Indeed, both IL-10 and IL-6 cytokines significantly decreased in the presence of pargyline upon 3h LPS exposure, indicating a direct contribution of MAO-dependent ROS formation.

Taken together these results, in human samples, point out new insights of MAO activity in phagocytic cells. We demonstrated that MAO is not only important for amine degradation but it contributes to M2 MΦ differentiation and polarization by its product H_2O_2. These results suggest the possibility to consider MAO as a pharmacological target to modulate MΦ differentiation and polarization, in particular upon pathological conditions. Indeed, it is well known how M2 MΦ are involved in fibrosis, cardiovascular remodelling, IR injury,[68, 101] as well as tumoral environment.[217] Thus, the possibility to modulate MΦ activity and differentiation can represent a great advantage to reduce inflammation-dependent damages upon pathological conditions.

9. BIBLIOGRAPHY

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