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Monoamine oxidase-dependent histamine catabolism accounts for post-ischemic cardiac redox imbalance and injury

Veronica Costiniti[a,1], Iolanda Spera[b,1], Roberta Menabò[a,c], Erika M. Palmieri[b,d], Alessio Menga[e], Pasquale Scarcia[b], Vito Porcelli[b], Rosanna Gissi[b], Alessandra Castegna[b,e,*], Marcella Canton[a,f,**]

[a] Department of Biomedical Sciences, University of Padova, Italy
[b] Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, Italy
[c] Neuroscience Institute, National Research Council of Italy (CNR), Padova, Italy
[d] The Cancer and Inflammation Program, National Cancer Institute-Frederick, Frederick, MD 21702, USA
[e] Hematology Unit, National Cancer Research Center, Istituto Tumori 'Giovanni Paolo II', Bari 70124, Italy
[f] Venetian Institute of Molecular Medicine (VIMM), Padova, Italy

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ABSTRACT

Monoamine oxidase (MAO), a mitochondrial enzyme that oxidizes biogenic amines generating hydrogen peroxide, is a major source of oxidative stress in cardiac injury. However, the molecular mechanisms underlying its overactivation in pathological conditions are still poorly characterized.

Here, we investigated whether the enhanced MAO-dependent hydrogen peroxide production can be due to increased substrate availability using a metabolomic profiling method. We identified N^1-methylhistamine -the main catabolite of histamine- as an important substrate fueling MAO in Langendorff mouse hearts, directly perfused with a buffer containing hydrogen peroxide or subjected to ischemia/reperfusion protocol. Indeed, when these hearts were pretreated with the MAO inhibitor pargyline we observed N^1-methylhistamine accumulation along with reduced oxidative stress. Next, we showed that synaptic terminals are the major source of N^1-methylhistamine. Indeed, *in vivo* sympathectomy caused a decrease of N^1-methylhistamine levels, which was associated with a marked protection in post-ischemic reperfused hearts. As far as the mechanism is concerned, we demonstrate that exogenous histamine is transported into isolated cardiomyocytes and triggers a rise in the levels of reactive oxygen species (ROS). Once again, pargyline pretreatment induced intracellular accumulation of N^1-methylhistamine along with decrease in ROS levels. These findings uncover a receptor-independent mechanism for histamine in cardiomyocytes.

In summary, our study reveals a novel and important pathophysiological causative link between MAO activation and histamine availability during pathophysiological conditions such as oxidative stress/cardiac injury.

1. Introduction

It is widely accepted that oxidative stress and mitochondrial dysfunction play a key role in cardiac injury [1–6]. Among the sources of reactive oxygen species (ROS), the mitochondrial enzyme monoamine oxidase (MAO) has been shown to significantly contribute to oxidative stress in cardiac dysfunction [7–11]. The role of this enzyme has been widely studied in the central nervous system for many years [12], whereas the impact of its activity in muscle function has been quite overlooked and considered only recently. Indeed, MAO pharmacological and genetic inhibition protects the heart in experimental models of cardiac reperfusion injury, decompensated hypertrophy and diabetic cardiomyopathy [7–11, 13]. In addition, MAO was recently found to mediate endothelial dysfunction in aorta [11, 14] and to contribute to

Abbreviations: DHE, Dihydroethidium; CD11b, cluster of differentiation molecule 11b; fam, famotidine; Gr-1, granulocytic marker 1; HDC, histidine decarboxylase; his, histidine; HNMT, histamine *N*-methyltransferase; IR, ischemia reperfusion; H2R, histamine receptor 2; HW, heart weight; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; MAO, monoamine oxidase; MRM, multiple reaction monitoring; MTR, MitoTracker Red CM-H2Xros; NE, norepinephrine; NMH, N^1-methylhistamine; NRVMs, neonatal rat ventricular myocytes; OCT3, organic cation transporters isoform 3; Parg, pargyline; PMAT, plasma membrane monoamine transporter; TH, tyrosine hydroxylase; Tm, tropomyosin; veh, vehicle; 6OH-DA, 6-hydroxy-dopamine

* Correspondence to: A. Castegna, Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, Italy.
** Correspondence to: M. Canton, Department of Biomedical Sciences, University of Padova, Italy.
E-mail addresses: alessandra.castegna@uniba.it (A. Castegna), marcella.canton@unipd.it (M. Canton).
[1] These authors contributed equally to the work.

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myofiber damage in muscular dystrophy [15, 16]. This flavoenzyme is located at the outer mitochondrial membrane and exists in two isoforms, A and B, with distinct substrate specificity and inhibitor sensitivity [12]. Pargyline is a selective irreversible inhibitor for both the isoforms -with higher affinity for MAO B- that has been safely used in clinics [12]. MAO catalyzes the oxidative deamination of biogenic amines (*i.e.* neurotransmitters) with production of aldehydes, ammonia and hydrogen peroxide (H_2O_2). Its physiologic role is well-established in brain, where it terminates neurotransmitter signaling [17, 18]. The amount of H_2O_2 generated by neurotransmitter oxidation is constantly removed by endogenous scavengers under physiological conditions. On the contrary, in pathological conditions MAO-driven H_2O_2 formation overcomes the cellular antioxidant defenses, potentially eliciting deleterious effects [12] as suggested in cardiac injury. However, the molecular mechanisms underlying the enhanced MAO activation in cardiac injury have been only partially elucidated [7–9]. This has prompted us to investigate the possibility that a large amount of substrates becomes available upon oxidative stress, thereby sustaining the increased MAO-mediated ROS production in cardiac injury.

In the present work, we identified and quantitated a new metabolic pathway sustaining MAO-related ROS production both in *ex vivo* and *in vitro* experimental models of oxidative stress. To this aim, we performed a first screening taking advantage of a simple but well-established model of oxidative stress in cardiovascular research, *i.e.* isolated mouse hearts perfused with H_2O_2 [19–21]. Application of H_2O_2 (0.01–1 mM) to isolated cells and intact organs appears to be directly relevant to biology, broadly mimicking the release of endogenous peroxides mediated by growth factors, as reviewed in [20]. Importantly, the study was extended to isolated mouse hearts subjected to post-ischemic reperfusion injury, a classical condition associated with oxidative and nitrosative stress [2, 22]. Additionally, we sought to determine the potential sources of these substrates in the whole heart and elucidate the underlying molecular mechanisms in isolated cardiomyocytes.

2. Results

2.1. Identification of the substrates that fuel MAO in perfused isolated hearts subjected to oxidative stress

Two different protocols of oxidative stress, performed in Langendorff-perfused mouse hearts, were considered in this study. Firstly, we exploited a simple protocol of 15 min-perfusion with high concentration of H_2O_2, to cause maximal oxidative stress by overriding the oxidant defenses (1 mM) [19, 20, 23, 24]. Fig. 1A shows that H_2O_2 perfusion induced a marked increase of ROS levels, as detected in heart cryosections by dihydroethidine staining. Interestingly, addition of pargyline to the perfusion buffer (before H_2O_2) caused a significant drop in these levels, indicating that ROS overproduction is mediated by MAO activity. In addition, the extent of oxidative stress in the same hearts was assessed by measuring the oxidation of myofilament proteins, which are ROS targets [25, 26]. Since we reported that tropomyosin is particularly susceptible to oxidation [23, 27, 28], we used it as a probe to assess oxidation of myofilament proteins. A representative anti-tropomyosin immunoblot displays a high molecular weight band in H_2O_2-perfused cardiac samples, which is due to dimer formation [28] (Fig. 1B). The degree of oxidation, as indicated by tropomyosin dimer to monomer ratio, decreased when pargyline was added before H_2O_2 perfusion (Fig. 1C). These results highlight the crucial role of MAO in this acute model of oxidative stress and are in line with previous studies demonstrating the relevance of this mitochondrial enzyme in several experimental models of cardiac and skeletal muscle diseases [7–10, 15].

2.2. NMH is a novel substrate for MAO in cardiac injury

In order to investigate the underlying mechanism, we tested the hypothesis that an increased amount of MAO substrates -amines- became available under oxidative stress conditions. Therefore, we developed a mass spectrometric method to identify and quantitate the cardiac "aminome", *i.e.* the set of all of the different amines in the isolated hearts. To assess the occurrence of substrate accumulation, we blocked their consumption by inhibiting MAO with pargyline. The comparison of the derivatized amine content following H_2O_2 perfusion in pargyline-*versus* vehicle-treated hearts allowed us to identify the accumulating amino-compounds, thus potential substrates of this enzyme (Fig. 2A and Supplemental Fig. 1). On the amines found to be accumulated in H_2O_2-perfused hearts when MAO is inhibited, a further level of quantification was achieved by using a LC-MS/MS method (Fig. 2B). Together with norepinephrine, typical MAO substrate, histamine and N^1-methylhistamine (NMH) unexpectedly accumulated in pargyline-treated H_2O_2-perfused hearts (Fig. 2C). NMH is the major intracellular catabolite of histamine, obtained by methylation of the imidazole ring in a reaction catalyzed by histamine *N*-methyltransferase (Fig. 2D) [29]. NMH is a MAO-B substrate whereas histamine is not, as assessed by an *in vitro* activity assay with the isolated enzyme (Supplemental Fig. 2). This is in accordance with pioneer studies performed in brain homogenates [30], that have been quite recently confirmed [31]. NMH

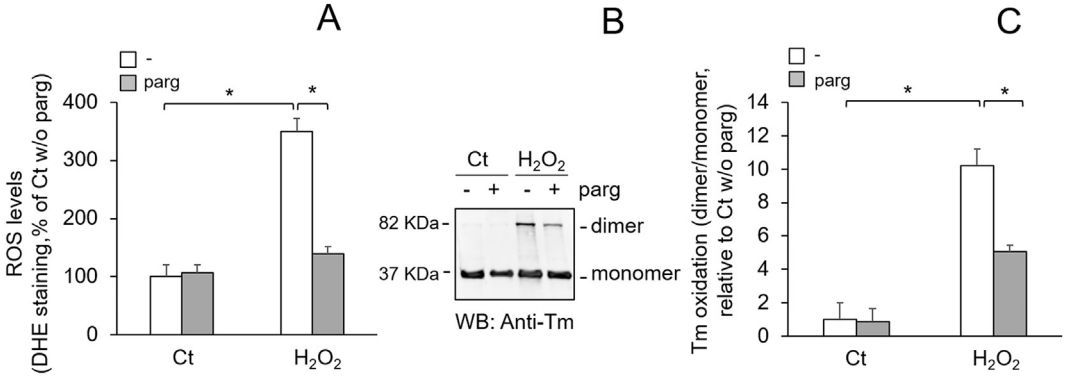

Fig. 1. MAO inhibition reduces oxidative stress in isolated mouse hearts perfused with H_2O_2. Isolated mouse hearts were Langendorff-perfused for 15 min in the absence (Ct) or presence of 1 mM H_2O_2. To test the effect of MAO inhibition the perfusions were performed in the absence or presence of 0.5 mM pargyline added to the perfusion buffer before H2O2 (n = 3 mice for each group). A) Oxidative stress was assessed by DHE staining (5 μM, 30 min) of heart cryosections and visualized by fluorescence microscopy. Quantification of DHE fluorescence showed a reduced ROS accumulation in H_2O_2-perfused hearts upon pargyline exposure; B) tropomyosin oxidation detected as the formation of dimers in heart samples denatured in non-reducing conditions (absence of β-mercaptoethanol). Representative Western blot of three independent experiments displayed a high molecular weight band (dimer) in H_2O_2-perfused samples that was reduced when pargyline was added to the perfusion buffer; C) quantitative analysis of tropomyosin oxidation, given by the ratio between the densitometric values of the dimer band and those of the corresponding monomer band. Parg, pargyline; Tm, tropomyosin; WB, Western blot. *P < 0.05. Values are the mean of at least three independent experiments.

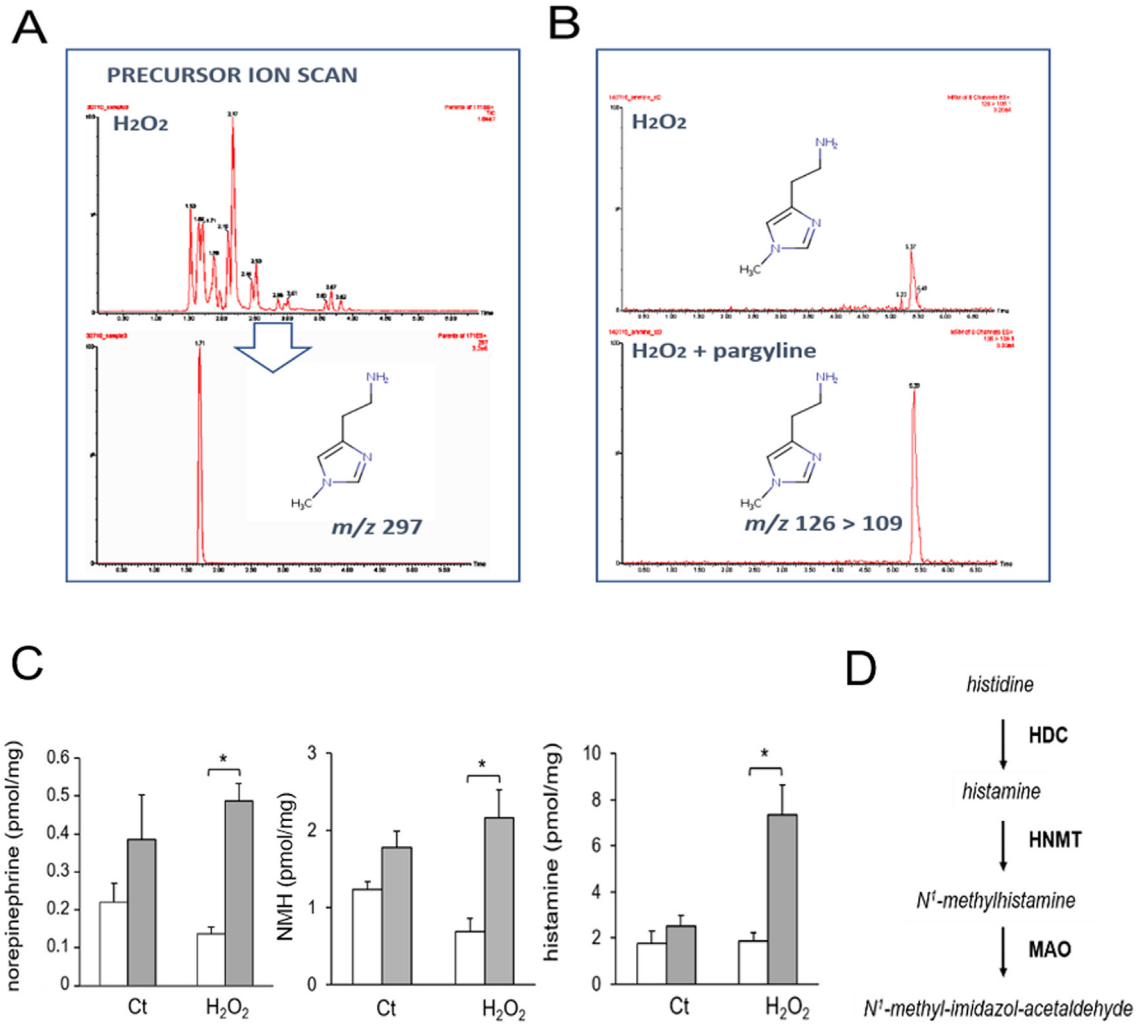

Fig. 2. Pargyline induces amine accumulation in H_2O_2 perfused mouse hearts. Isolated mouse hearts were perfused in the absence or presence of H_2O_2 as described in Fig. 1 (n = 3 mice for each group). Amines extracted from these hearts were identified and quantitated by mass spectrometry. A) Representative graph (*top*) of the precursor ion scan amine profiling of H_2O_2-treated perfused mouse hearts in which the *derivatized* N^1-methylhistamine (NMH) peak (*m/z* 297, 126 + 171) was extracted (*bottom*); B) representative graph of NMH quantified by MRM in both H_2O_2 (*top*) and H_2O_2-pargyline samples (*bottom*); C) quantitative analysis of NMH, histamine, and norepinephrine *P < 0.05. Parg, pargyline. Values are the mean of at least three independent experiments. D) Scheme for intracellular histamine metabolism. Histidine decarboxylase (HDC) synthesizes histamine by decarboxylation of the amino acid histidine. Histamine can be methylated to NMH by histamine *N*-methyltransferase (HNMT). NMH is oxidatively deaminated to the corresponding aldehyde by MAO.

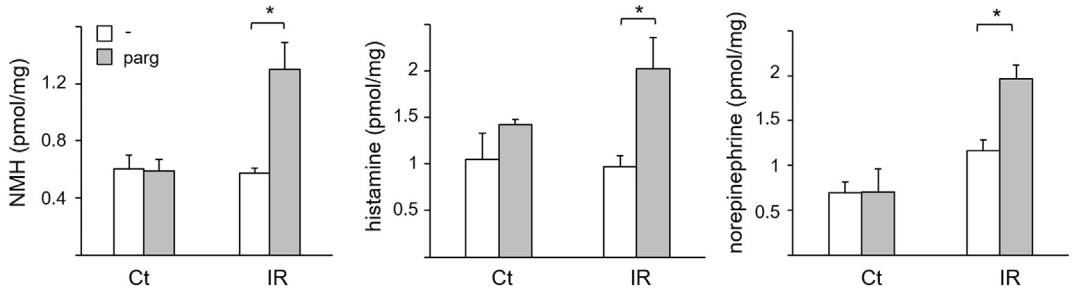

Fig. 3. Pargyline induces accumulation of histamine metabolites in hearts subjected to post-ischemic reperfusion. Isolated mouse hearts were normoxically perfused (Ct) (n = 3 mice) or subjected to 40 min-ischemia followed by 15 min-reperfusion (IR) in the absence or presence of 0.5 mM pargyline (n = 6 mice for each group), added to the perfusion buffer before the IR protocol. N^1-methylhistamine, histamine and norepinephrine were quantitated by MRM analysis. Parg, pargyline. *P < 0.05. Values are the mean of at least three independent experiments.

was identified as an additional and significant MAO substrate in the heart. Indeed, upon MAO inhibition, its levels increase of 1.5 pmoL/mg in H_2O_2-perfused hearts, as compared to a rise of 0.4 pmoL/mg for norepinephrine (Fig. 2C). Therefore, NMH is a novel metabolite which can support the enhanced MAO activity in our model of cardiac injury.

Next, we exploited our "aminome" profiling approach to seek for MAO substrates in post-ischemic reperfused hearts, a classical model of cardiac injury. Together with norepinephrine, NMH accumulated also in post-ischemic reperfused hearts when MAO was inhibited (Fig. 3), further supporting the relevance of histamine metabolism in MAO-

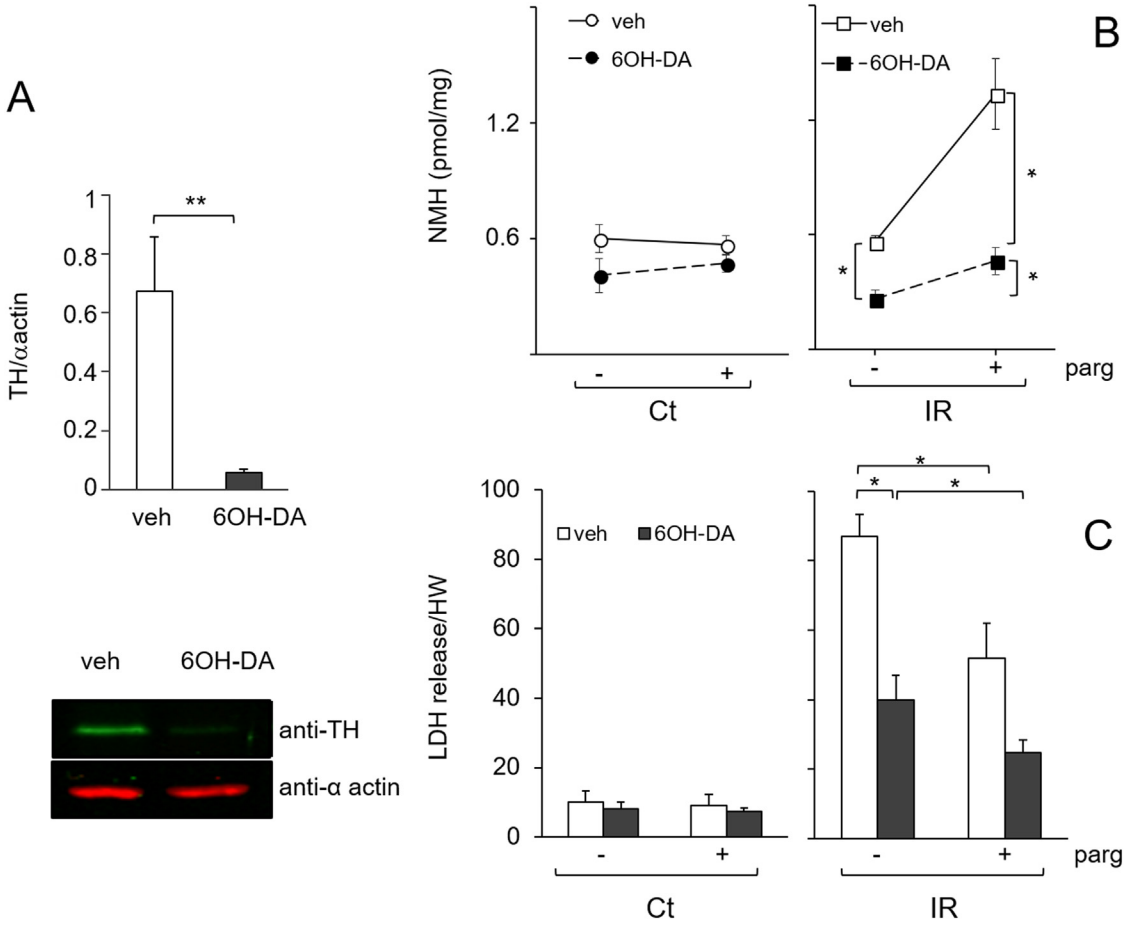

Fig. 4. *In vivo* sympathectomy reduces N^1-methylhistamine levels and loss of viability in post-ischemic reperfused hearts. Mice were injected with 6-hydroxydopamine to induce denervation (6OH-DA, 0.1 mg/g mouse weight) or with vehicle (veh), sacrificed after 24 h and subjected to normoxic perfusion (Ct) (n = 8 mice) or post-ischemic reperfusion (IR) (n = 12 mice) as described in Fig. 3. Both the perfusion protocols were performed in the absence and presence of 0.5 mM pargyline (n = 4 mice for each group in normoxic conditions, n = 6 mice for each post-ischemic reperfusion group). A) Heart samples from veh- or 6OH-DA-treated mice were assessed by immunoblot analysis for anti-tyrosine hydroxylase (TH) antibody to test the efficacy of denervation. Band intensities were quantified by densitometry and normalized to those of α-actin (*upper panel*) to check protein loading, showing a marked denervation. Lower panel: representative Western blot; B) denervation by 6OH-DA induced a significant drop of N^1-methylhistamine (NMH) levels in IR samples, highlighting the contribution of synaptic terminals. Pargyline administration caused accumulation of this amine not only in sham hearts undergoing IR but also in denervated hearts, although at a lower extent; C) myocardial loss of viability, as detected by LDH released in the effluents, was reduced in hearts from 6OH-DA-treated mice. Furthermore, pargyline showed a protective effect in hearts from both veh- and 6OH-DA-treated mice. Data are expressed as LDH activity in the effluent normalized to heart weight (HW). *P < 0.05, **P < 0.01. 6OH-DA, 6-hydroxydopamine; veh, vehicle; LDH, lactate dehydrogenase.

mediated cardiac injury. In parallel, MAO inhibition reduced ROS levels in post-ischemic reperfused hearts, as detected by dihydroethidine staining (Supplemental Fig. 3) and by the degree of tropomyosin oxidation [32].

2.3. Sympathetic nerves as relevant sources of histamine and NMH

Since we established the relevance of NMH in our experimental models, we aimed at identifying its cellular sources. Based on previous studies demonstrating the role of histaminergic neurons in the heart [33–35], we hypothesized that NMH originates from the histamine stored in the synaptic terminals. Indeed, histamine co-localizes with norepinephrine in neurons innervating the cardiovascular system [33, 35]. To this aim, mice were subjected to cardiac sympathetic denervation by means of 6-hydroxydopamine (6OH-DA), a well-established procedure that induces peripheral autonomic neuron degeneration without affecting central adrenergic neurons [36], and with well-characterized, minimal consequences on resting blood pressure and cardiac haemodynamics [37, 38]. After 24 h from the injection, the isolated hearts were subjected to post-ischemic reperfusion. The efficacy of the denervation procedure was assessed by evaluating the decrease in tyrosine hydroxylase protein levels, a marker for functional synaptic terminals (Fig. 4A). In line with the literature, we observed a marked ablation of cardiac sympathetic nerve terminals 24 h after the 6OH-DA intraperitoneal injection [39]. No significant differences in heart weight were observed (heart weight/body weight was $6.45 \pm 1.2 * 10^{-4}$ for denervated *versus* $6.6 \pm 1.2 * 10^{-4}$ for vehicle-treated mice). Denervation induced a decrease by 53% of NMH levels in post-ischemic hearts (Fig. 4B, *right panel*), along with a drop in norepinephrine contents, but not of histamine (Supplemental Fig. 4). Thus, our findings suggest that synaptic terminals represent a relevant source for MAO substrates in post-ischemic reperfused hearts. Next, to test whether additional sources can contribute to MAO activation, we denervated mice and then performed cardiac post-ischemic reperfusion in the absence or presence of pargyline. The rationale is based on the idea that if MAO-inhibition causes NMH accumulation even in the absence of nerve terminals, the contribution from other sources should be considered. Indeed, NMH levels increased upon pargyline treatment also in denervated hearts undergoing post-ischemic reperfusion (Fig. 4B *right panel*), although at a lower extent as compared to hearts from vehicle-treated mice. This suggests the contribution of additional sources besides nerves to the NMH pool. On the contrary, pargyline did

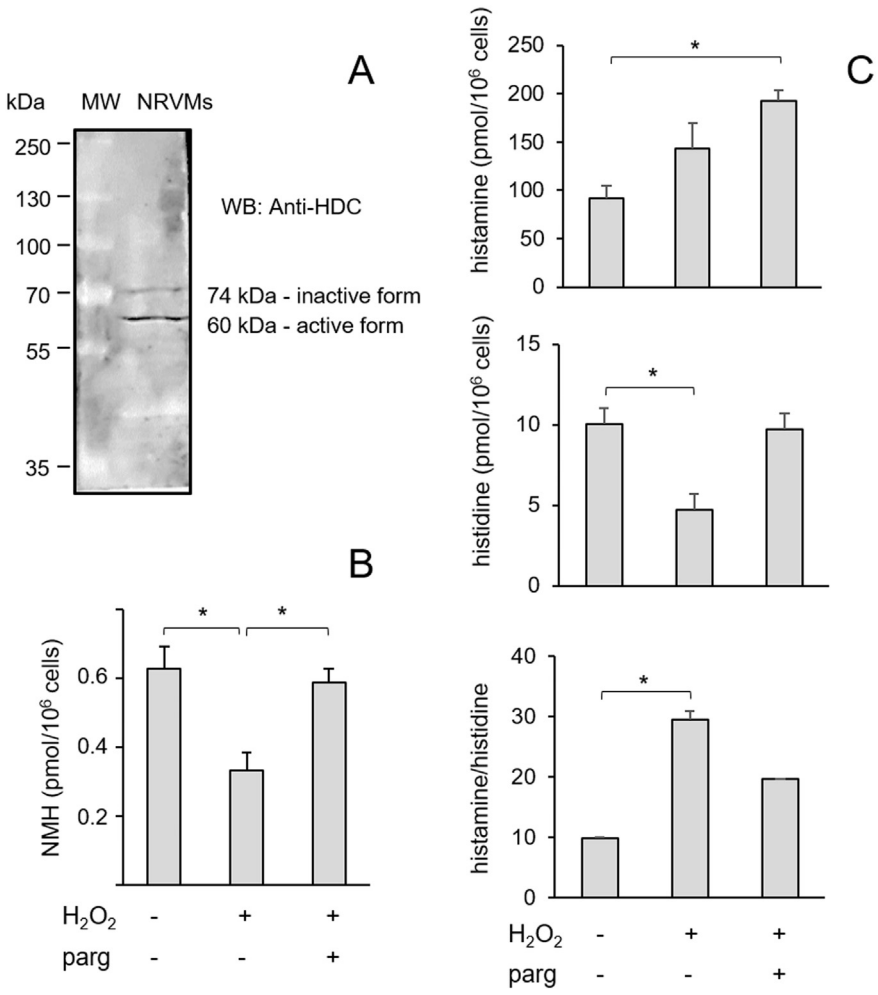

Fig. 5. Neonatal rat cardiac myocytes synthesize MAO substrates. A) Neonatal rat ventricular cardiomyocytes (NRVM) express histidine decarboxylase (HDC), as detected by Western blot with anti-HDC antibodies. Two bands were observed, corresponding to the inactive and the active form of the enzyme (74 kDa and 60 kDa, respectively); B) N^1-methylhistamine (NMH) and C) histamine and histidine levels were measured by MRM analysis in cardiomyocytes exposed to 100 μM H_2O_2 for 1 h in the absence or presence of 100 μM pargyline (as a 15 min pretreatment). NMH accumulated upon MAO inhibition, as in the *ex vivo* model. *P < 0.05. Values are the mean of at least three independent experiments. Parg, pargyline; WB, Western blot, MW, molecular weights.

not modify the levels of norepinephrine in denervated hearts, suggesting that nerve terminals were its main source (Supplemental Fig. 4).

Notably, cardiac denervation caused a marked protection of the hearts, as detected by a reduced release of lactate dehydrogenase (LDH) (Fig. 4C, *right panel*) and lower ROS levels (Supplemental Fig. 5) in post-ischemic reperfused hearts. These findings confirmed the protective efficacy of the procedure, according to previous studies summarized in [40], and showed that depletion of MAO substrates is associated to cardioprotection. In parallel, pargyline remarkably reduced the release of LDH from post-ischemic reperfused hearts not only in vehicle-treated (as demonstrated in [32]) but also in denervated mice (Fig. 4C, *right panel*). The MAO inhibition-driven protection observed in denervated hearts, paralleled by NMH accumulation, further lends support to the presence of other sources contributing to cardiac damage. This evidence prompted us to select cardiomyocytes as potential additional source. Indeed histamine, NMH and epinephrine were detected in neonatal rat ventricular myocytes by mass spectrometry (not shown). Histidine decarboxylase (HDC), which catalyzes the conversion of histidine into histamine, was detected for the first time in our cardiomyocyte preparation (Fig. 5A), indicating that these cells can synthesize their own histamine. The bands migrating at about 74 and 60 kDa correspond to the precursor and the active form of the enzyme, respectively [41]. Thus, we hypothesized that cardiomyocytes could produce MAO substrates *per se* under conditions promoting oxidative stress, thereby sustaining MAO activity. To this aim, amine levels were quantified in cardiomyocytes exposed to H_2O_2 for 1 h in the absence or presence of pargyline. NMH accumulated upon H_2O_2 exposure when MAO was inhibited as in the *ex vivo* model, further confirming an important role for this substrate (Fig. 5B). Administration of H_2O_2 elicited also an increase of the histamine levels - which became significant when MAO was inhibited (Fig. 5C, *upper panel*) - along with a drop in the intracellular levels of histidine (Fig. 5C, *medium panel*). Thus, when histamine content was normalized to intracellular histidine levels, the histamine/histidine ratio was 3-fold higher in H_2O_2-treated myocytes *versus* sham (Fig. 5C, *lower panel*) indicating an increased histidine catabolism in H_2O_2-treated myocytes. These *in vitro* data support the hypothesis that histidine decarboxylase might be activated by H_2O_2 according to previous studies [42], thereby contributing to a rise in histamine and, in turn, NMH levels.

2.4. Role of MAO in isolated cardiomyocytes exposed to histamine

Lastly, we tested whether external applied histamine (simulating the histamine released from nerve endings to the neurojunctional area) could enter cardiomyocytes, activate MAO and promote ROS formation. Neonatal rat ventricular myocytes were incubated with histamine (50 μM, 1 h), which triggered a marked rise in ROS levels (Fig. 6A), as detected by a redox-sensitive probe, MitoTracker Red (MTR) in the reduced form (CM-H2Xros). This was suppressed by both pargyline (Fig. 6A) and rasagiline (Supplemental Fig. 6), further supporting a pivotal role of MAO B in cardiac histamine catabolism. Upon histamine exposure we observed an intracellular rise of its levels, indicating that it was transported into cardiomyocytes (Fig. 6B upper panel). Importantly, the uptake was paralleled by increased levels of NMH, which became significant when pargyline inhibited its MAO-driven consumption (Fig. 6B lower panel).

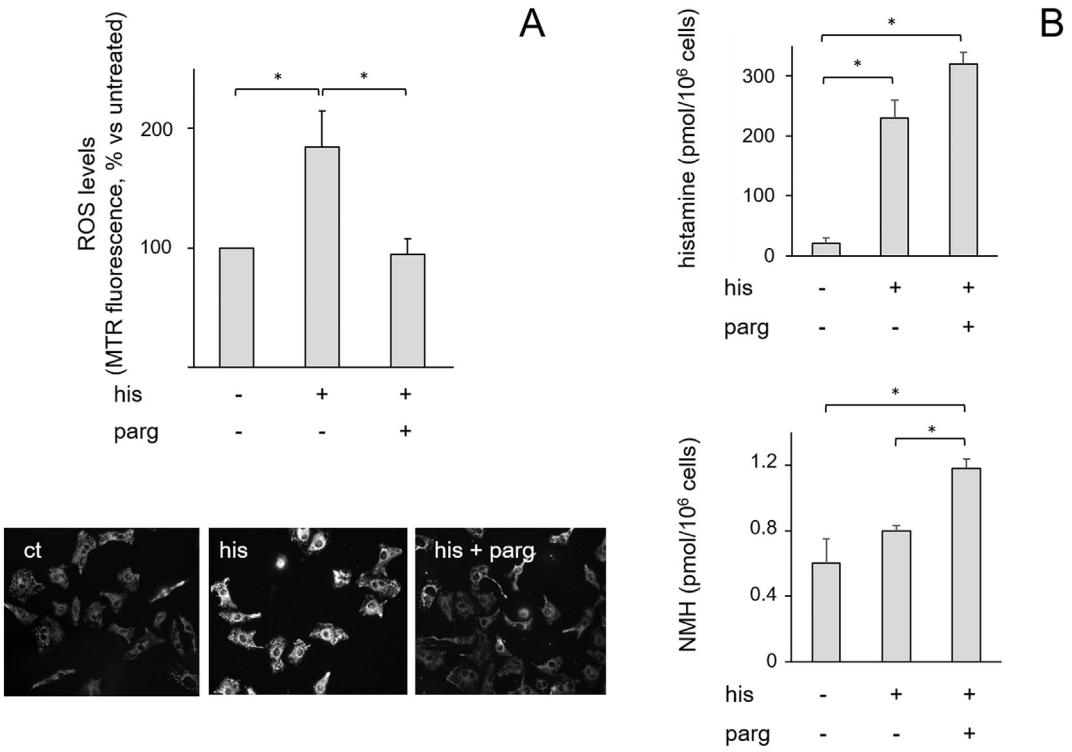

Fig. 6. Histamine induces a MAO-mediated rise in ROS levels in neonatal rat cardiac myocytes. A) ROS levels were assessed by measuring MitoTracker Red CM-H2Xros fluorescence (MTR, 20 nM) after cardiomyocyte treatment with 50 μM histamine for 1 h in the absence or presence of 100 μM pargyline (as a 15 min pretreatment). MTR fluorescence quantification (*upper panel*) shows that histamine induced an increase of ROS levels as compared to the untreated cells (ct), which is suppressed by pargyline administration. Representative photomicrographs of cardiomyocytes (*lower panel*) treated as described (magnification ×63); B) histamine and N^1-methylhistamine (NMH) levels were quantitated by MRM analysis. Exogenous histamine is transported into myocytes and causes NMH increase, which becomes significant in the presence of pargyline. These data support a role for histamine catabolism in isolated cardiomyocytes and its relation with MAO activity. * $P < 0.05$. Values are the mean of at least three independent experiments. Parg, pargyline.

These results show that histamine acts through a receptor-independent mechanism, promoting a rise of the intracellular ROS levels associated to the MAO-mediated NMH consumption. However, to exclude that this effect could also occur through a pathway dependent on stimulation of the histamine receptor 2 (H2R), which is expressed on cardiomyocyte plasma membrane [34], we treated cardiac myocytes with famotidine, a specific H2R antagonist [34, 43]. Famotidine did not significantly reduce the histamine-dependent rise in ROS levels (Supplemental Fig. 7), suggesting that the H2R does not play a major role in this mechanism.

3. Discussion

The present study provides three key findings: (*i*) NMH is a crucial cardiac substrate which fuels MAO and elicits ROS production in our models of cardiac injury; (*ii*) synaptic terminals innervating the heart are a relevant source of NMH; (*iii*) endogenous/exogenous histamine promotes ROS formation in isolated cardiomyocytes in a MAO-dependent manner.

Although MAO is one of the major sources of ROS in the myocardium, the molecular mechanisms that are responsible for its enhanced activity in pathological conditions are only partially clarified [44]. Elucidation of how MAO contributes to cardiac dysfunction would be of paramount pathogenic and therapeutic relevance.

3.1. NMH is a novel substrate that enhances MAO activity in isolated perfused hearts undergoing cardiac injury

The present study uncovers the relevance of NMH, the main product of the intracellular histamine catabolism, as a major player contributing to MAO activation in the heart upon oxidative stress. Although the enzymes responsible for histamine metabolism were identified in the heart in the nineties [45], the role of NMH in cardiac injury is novel. In fact, previous studies investigating MAO activation in heart dysfunction have been essentially focused on the role of serotonin and norepinephrine, classical substrates for the enzyme. Specifically, serotonin has been reported to play a crucial role in post-ischemic reperfused hearts and in cardiac hypertrophy [7, 46]. Catecholamines are also well known to contribute to cardiovascular diseases, increase in chronic heart failure, hypertension, and be coupled to ROS signaling [47–49]. The impact of MAO-A on neurotransmitter availability has been previously characterized in a murine model of heart failure [8]. In particular, the enhanced MAO-A activity was found to be coupled to intramyocardial availability of norepinephrine, resulting in ROS increase and cardiac dysfunction. However, the study was limited to the assessment of the cardiac amounts of the more likely candidates, *i.e.* norepinephrine, serotonin and their catabolites. Here, we exploited an unbiased metabolomic approach ("the aminome") which allowed us to identify NMH as a relevant additional substrate that fuels MAO. This unexpected finding represents a major achievement of the present study, highlighting a novel relationship between NMH and MAO activation in the heart. These results imply that the profound injury induced by H_2O_2 administration or post-ischemic reperfusion is not due to a direct action. Indeed, these protocols appear to trigger an amplification pathway whereby the increase in MAO activity due to a larger substrate availability is the end-effector of the initial oxidative stress. Furthermore, these data highlight an important difference between NMH and the classical MAO substrate, norepinephrine. At variance with NMH, the expected decrease in norepinephrine levels following denervation is not rescued by MAO inhibition, suggesting that its pool is mainly maintained by the neurons, with negligible contribution from mast or other cells. With this respect, it is known that mast cells do not

release norepinephrine directly, but might contribute only marginally to its pool through renin-mediated stimulation of surrounding cells [50]. The histamine-NMH axis represents then a novel route supporting MAO activity.

3.2. Synaptic terminals innervating the heart are crucial sources of MAO substrates in ex vivo post-ischemic reperfused hearts

Endogenous histamine is a determinant player in many cardiovascular pathologies, such as atherosclerosis and chronic heart failure [51–53] and its plasmatic levels are increased in patients with ischemic heart diseases [34, 54]. Despite its widely recognized role in ischemic hearts, the underlying molecular mechanisms are still matter of discussion, especially with respect to the origin of endogenous histamine. Previous reports suggested that mast cells are a source of endogenous histamine in cardiovascular disorders [55]. Indeed, in an ischemic state and type 1 diabetes, cardiac mast cells degranulate and release histamine, renin and several molecules which induce arrhythmogenesis, fibrosis and inflammation [13, 50, 56]. Additionally, cardiac sympathetic nerves also express HDC, the rate-limiting enzyme in the synthesis of histamine [57]. Indeed almost 40% of neurons in human cardiac ganglia are immunoreactive for histamine and HDC [57]. Under acute myocardial ischemic conditions, cardiac sympathetic nerves release neurotransmitters in large amounts (among which histamine), inducing ventricular arrhythmia [33]. The released histamine is reported to facilitate the arrhythmogenic effect of simultaneously released norepinephrine [33]. Intriguingly, here we provided evidence that the synaptic terminals innervating the heart are a significant source of NMH in our experimental models, that was previously ignored. As expected, the decrease in its levels following denervation (Fig. 4B) was paralleled by a drop of norepinephrine content (Supplemental Fig. 4). The lower availability of these substrates was paralleled by a protective effect, suggesting a link between MAO activation and cardiac damage. In addition, our findings suggest that other sources can contribute to NMH, thus to MAO activity, such as immune cells (*i.e.* mast cells or macrophages) and cardiomyocytes. This is substantiated by the finding that histamine levels do not significantly decrease in denervated mice undergoing I/R (Supplemental Fig.4). Notably, we found that isolated cardiomyocytes are equipped with their own HDC and contain both histamine and NMH (see Fig. 5). Further studies will extend this novel metabolomic approach to more complex *in vivo* models of cardiac damage. It will be interesting to assess the contribution of immune cells [58], as they can be recruited massively from blood at variance with our *ex vivo* perfused hearts. With this respect, Deshwal et al. [13] showed that, in their model of type 1 diabetes, MAO-dependent ROS formation could trigger mast cell degranulation thereby contributing to extracellular matrix remodeling, consequent LV fibrosis and dysfunction. They reported that cardiomyocytes represent a target of the inflammatory response confirming our model. Indeed, our study suggests that cardiomyocytes are a target of several *stimuli* such as histamine and MAO substrates released by different cytotypes (*i.e.* sympathetic nerves). In another study the $CD11b^+$ $Gr\text{-}1^+$ myeloid cells have been identified as the major cellular source of endogenous histamine in the hematopoietic system, due to their high HDC expression [59]. Furthermore Xu et al. [60] reported that hypercholesterolemia increases the number of HDC-expressing $CD11b^+Gr\text{-}1^+$ myeloid cells, which may play a critical role in atherogenesis. They show that atherosclerotic plaques from $Apoe^{-/-}$ mice display HDC^+ myeloid cells, and that histamine promotes the differentiation of macrophages from $CD11b^+$ myeloid cells and the formation of foam cells. These findings open the interesting possibility that MAO activity might be relevant during inflammation and that histamine production might be a shared mechanism that, from one side promotes and sustains foam cells formation and atherosclerotic plaques, from the other side induces cardiac injury by providing MAO substrates. Studies in our laboratories are going in this direction.

3.3. Histamine-dependent ROS formation in isolated cardiomyocytes is mediated by MAO

Histamine is a pleiotropic amine that promotes different physiological processes through its specific four G protein-coupled receptors [61, 62], acting as a neurotransmitter [33]. However, its physiological actions are mediated not only by receptor-dependent but also by receptor-independent mechanisms. The latter involve the uptake of histamine, followed by its inactivation through histamine *N*-methyltransferase [63]. This dual mechanism, receptor-dependent and -independent, has been previously studied with catecholamines and serotonin not only in neurons but also in myocytes [7, 8, 46, 64]. The general *consensus* is that, when these amines are in excess, they enter not only neurons but also myocytes, where they are cleared by MAO. With this respect, it has been previously proposed that, under pathological conditions such as cardiac failure, a defective re-uptake of catecholamines in neurons causes a “spillover”, so that they can be transported into cardiomyocytes and removed by MAO [8].

Our *in vitro* findings showed that also exogenous histamine is transported into isolated cardiomyocytes and elicits a rise of ROS levels mediated by MAO. Indeed, histamine can enter through the organic cation transporters, especially the isoform 3 (OCT3, solute carrier family 22 member 3), that is expressed in the heart [65], but also through the plasma membrane monoamine transporter (PMAT), although the latter appears to be more relevant for the central nervous system [66]. Interestingly, Zhu and coworkers showed that the key role of histamine during brain ischemia was mediated by the OCT3 transporter since genetic ablation of *oct3* ameliorated ischemic brain damage [67]. The protection of *oct3*-deficient mice to brain injury can now be interpreted in light of the present results, suggesting that impairment of the histamine influx into neuronal cells might be beneficial since it prevents MAO-driven ROS formation. Further studies will elucidate this issue. Taken together, our *in vitro* data showed that histamine induces a rise in ROS levels, which occurs independently of receptor stimulation.

4. Conclusions

Taken together, these findings indicate that histamine metabolism provides an important contribution to regulate MAO activity, by increasing the availability of NMH. This highlights an unexpected relationship between histamine catabolism and MAO activation in cardiac injury. Remarkably, the critical contribution of synaptic terminals in promoting MAO-mediated cardiac damage underlies the importance of the extra-myocyte origin of MAO substrates and suggests a significant trafficking of MAO substrates between myocytes and non-myocyte cells in the heart. In summary, we have provided a novel mechanism that contributes to explain how MAO activity is enhanced when the heart is subjected to oxidative stress and that can be exploited for future therapeutic purposes.

5. Materials and methods

5.1. Isolated heart perfusion

All aspects of animal care and experimentation were performed in accordance with Italian regulations concerning the care and use of laboratory animals and were approved by the Ethical Committee of the University of Padova. Male C57BL/6J mice of 4–5 months (30 g) were sacrificed by cervical dislocation. Hearts were rapidly excised and perfused with bicarbonate buffer gassed with 95% O_2–5% CO_2 at 37 °C (pH 7.4). Perfusion was performed in the non-recirculating Langendorff mode, as previously described [23]. The bicarbonate 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 10.0 glucose. Hearts were treated as follows ($n \geq 5$/group): after a 10 min-equilibration, they were either perfused under normoxic conditions in the absence or presence of 1 mM H_2O_2 for

15 min, or subjected to 40 min of global ischemia and 15 min of reperfusion (IR). The effect of MAO inhibition was examined by dissolving pargyline to a final concentration of 0.5 mM in the perfusion buffer (10 min before the protocols described above) and by maintaining it throughout the protocol. The concentration of inhibitor was selected according to the literature [68, 69]. In addition, 0.5 mM was determined as the concentration of pargyline that gave maximal protection in H_2O_2-perfused rat hearts (not shown). The perfusion protocols were terminated by freezing hearts in liquid nitrogen. Samples were stored in liquid nitrogen until analysis.

5.2. Pharmacological sympathectomy

Pharmacological sympathectomy was obtained by intraperitoneally injecting mice with 6-hydroxy-dopamine (6OH-DA, 0.1 mg/g mice weight, freshly dissolved in 0.9% NaCl and 0.1% ascorbic acid) [36]. Control mice were injected with vehicle (0.9% NaCl and 0.1% ascorbic acid). Animals (n = 5–9/group) were sacrificed 24 h after injection and their hearts were isolated and perfused under normoxic conditions or subjected to IR in the absence or presence of pargyline, as described in "Isolated heart perfusion". Afterwards, hearts were frozen for the following analysis. The efficacy of chemical denervation was assessed through Western blot analysis for every 6OH-DA-treated heart. Anti-tyrosine hydroxylase antibody (Sigma) was used as typical marker to target synaptic terminals.

5.3. Measurement of lactate dehydrogenase activity

To determine the amount of lactate dehydrogenase (LDH) released from the perfused hearts, coronary effluents were collected during the 15 min of reperfusion. LDH activity was determined in the effluents by measuring the changes of NADH absorbance at 340 nm according to a classical procedure [70]. The values were normalized to the weight of each heart.

5.4. Measurements of oxidative stress

5.4.1. Dihydroethidium (DHE) staining

DHE is oxidized by ROS, forming ethidium bromide, which emits red fluorescence when intercalates with DNA [71]. Heart cryosections (10 μm thick) were incubated with 5 μM DHE (Sigma) for 30 min at 37 °C in degassed PBS, washed twice in PBS, mounted and visualized using an inverted microscope Leica DMI6000B, a 10× objective and appropriate emission filters as previously described [15]. All the procedure was carried out in dark. Data were acquired and analyzed using Metamorph software (Universal Imaging). Tropomyosin oxidation. Sarcomeric protein enriched fraction was obtained in the presence of protease inhibitors, and the entire procedure was performed at 4 °C as described [27]. Briefly, 15–20 mg of frozen ventricles were homogenized in ice-cold buffer containing 25 mM imidazole and 5 mM EDTA (pH 7.2), added with 1× protease inhibitor mix (Roche). Just before use, the solution was stirred under vacuum and then bubbled with argon to maximally reduce oxygen tension. For immunoblotting analyses, 12 μg protein per lane were loaded in non-reducing conditions on 12% SDS-polyacrylamide resolving gels. After electroblotting, membranes were probed with anti-tropomyosin monoclonal antibody (CH1 clone, Sigma). Quantitative analysis of the degree of tropomyosin oxidation was performed on the densitometric values of the Western blot bands. The density of the band with higher molecular weight (reflecting dimer formation) was normalized to the main band (monomer) as previously described [28].

5.4.2. Mitochondrial ROS in isolated cardiomyocytes

They were detected using the fluorescent probe MitoTracker Red in the reduced form (MTR, MitoTracker Red CM-H_2XRos, Thermo Fisher Scientific). Neonatal rat cardiomyocytes were washed after the treatments and then loaded for 30 min with 20 nM MTR. All the steps were carried out at 37 °C with 5% CO_2. After washing, each analyzed coverslip fluorescence intensity was calculated as the mean fluorescence of all the selected regions of interest. Experiments with the different agents as described above were always performed in comparison with the irrespective controls. Images were collected using an inverted fluorescence microscope Leica DMI6000B equipped with a xenon lamp and a 12-bit digital cooled CCD camera. Fluorescence emission was monitored by using 568 ± 25 nm excitation and 585 nm longpass emission filter setting. Images were collected with an exposure time of 100 ms by using a 63× oil-immersion objective. Data were acquired and analyzed using Metafluor software (Universal Imaging).

5.5. Western blot analysis

Total lysates were prepared in SDS Laemmli buffer as described [23] and then loaded in 12% SDS-polyacrylamide resolving gels. The membranes were saturated with fat-free milk 5% in TBS (Tris-HCl 50 mM, NaCl 150 mM, pH 7.5) Tween 0.1% for 1 h at room temperature and probed with antibodies against HDC, tyrosine hydroxylase (TH) and α-sarcomeric actin (Sigma). Signals were amplified and visualized with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and enhanced chemiluminescence, or fluorochrome-conjugated secondary antibodies (LICOR) detected by the Odyssey Infrared Imaging System (LICOR Biosciences). Images of the acquired Western blots were analyzed using the ImageJ software.

5.6. Neonatal rat ventricular myocytes studies

Ventricular myocytes were isolated from 1- to 3-day-old Wistar rats, as previously described [72]. In brief, the hearts were removed, and the ventricles were minced in Hank's Balanced Solution (HBSS, Sigma). These tissue fragments were digested overnight at 4 °C by trypsin dissociation (Thermo Fisher Scientific). The day after cells were isolated by sequential digestions with type II collagenase (Thermo Fisher Scientific), filtered and preplated to enrich the myocyte fraction. The nonadherent myocytes were plated at a concentration of 5×10^5 cells/mL onto 0.1% gelatin-coated (porcine gelatin, Sigma) surfaces in modified essential medium (MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 10 units/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific), 5-bromo-2′-deoxyuridine (Sigma), 0.1% non-essential aminoacids (Thermo Fisher Scientific) and 1× Insulin Transferrin Selenium (Thermo Fisher Scientific). The final myocyte cultures contained > 90% cardiac myocytes at partial confluence, as detected by immunostaining with α-sarcomeric actin antibody (Sigma). Experiments were performed on d 3–6 of culture. Cells were rinsed and incubated for 1 h at 37 °C in serum-free medium containing H_2O_2 or histamine in the presence or the absence of the MAO inhibitor, pargyline (as a 15 min pretreatment).

5.7. Sample preparation and LC-MS/MS analysis

Heart samples were weighted, suspended in 500 μL of 50% (v/v) MeOH/water (containing formic acid, ascorbic acid and tris(2-carboxyethyl)phosphine (TCEP) to prevent amine oxidation) and homogenized using a mechanic homogenizer. Homogenized samples were then centrifuged at 13,200 rpm for 10 min at 4 °C. The aqueous fraction was separated and transferred to a new vial and derivatized or directly analyzed. Neonatal rat ventricular myocytes (5×10^6 cells) were extracted similarly and centrifuged at 13,200 rpm for 10 min at 4 °C.

For the amine profiling (aminome) a 10 μL aliquot of each sample was added to 80 μL of borate buffer (200 mM, pH = 8.8 at 25 °C). The resulting solution was vortexed and then 20 μL of 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC reagent, Waters), was added to derivatize the NH_2-group and immediately vortexed. The

samples were heated with shaking at 55 °C for 10 min then centrifuged and transferred to vials. Derivatized samples were analyzed in a precursor scanning mode, by selecting as precursor ions those producing the 171.1 *m/z* fragment ion with a collision energy set up 20 V within the mass range of 100–1000 *m/z* as described [73].

For amines displaying increased levels in pargyline-treated compared to untreated samples, a further multiple reaction monitoring (MRM) method was set up and run on underivatized samples in which selected ions were monitored in the positive mode using the following MRM transitions: 111.80 > 94.90 *m/z* for histamine, 126.00 > 109.10 *m/z* for NMH, 170.20 > 107.20 *m/z* for norepinephrine. In all cases samples were analyzed with an Acquity UPLC coupled to a Quattro Premier Spectrometer (Waters) [74–76]. Injection volumes of 5 μL of samples or standards were used. Chromatographic separation was achieved by using a HSS-T3 column and the flow was set at 0.3 mL/min.

5.8. Statistics

Data entry and all analyses were performed in a blinded fashion. Results are shown as means ± SEM. Statistical significance was calculated by two-tailed unpaired *t*-test or ANOVA test, with Bonferroni *post hoc* test and considered statistically significant as follows: $^*P < 0.05$.

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