Monoamine Oxidase A–Mediated Enhanced Catabolism of Norepinephrine Contributes to Adverse Remodeling and Pump Failure in Hearts With Pressure Overload


Rationale: Monoamine oxidases (MAOs) are mitochondrial enzymes that catabolize prohypertrophic neurotransmitters, such as norepinephrine and serotonin, generating hydrogen peroxide. Because excess reactive oxygen species and catecholamines are major contributors to the pathophysiology of congestive heart failure, MAOs could play an important role in this process.

Objective: Here, we investigated the role of MAO-A in maladaptive hypertrophy and heart failure.

Methods and Results: We report that MAO-A activity is triggered in isolated neonatal and adult myocytes on stimulation with norepinephrine, followed by increase in cell size, reactive oxygen species production, and signs of maladaptive hypertrophy. All of these in vitro changes occur, in part, independently from α- and β-adrenergic receptor–operated signaling and are inhibited by the specific MAO-A inhibitor clorgyline. In mice with left ventricular dilation and pump failure attributable to pressure overload, norepinephrine catabolism by MAO-A is increased accompanied by exacerbated oxidative stress. MAO-A inhibition prevents these changes, and also reverses fetal gene reprogramming, metalloproteinase and caspase-3 activation, as well as myocardial apoptosis. The specific role of MAO-A was further tested in mice expressing a dominant-negative MAO-A (MAO-Aneo), which were more protected against pressure overload than their wild-type littermates.

Conclusions: In addition to adrenergic receptor–dependent mechanisms, enhanced MAO-A activity coupled with increased intramyocardial norepinephrine availability results in augmented reactive oxygen species generation, contributing to maladaptive remodeling and left ventricular dysfunction in hearts subjected to chronic stress. (Circ Res. 2010;106:193-202.)

Key Words: monoamine oxidase A ■ congestive heart failure ■ oxidative stress ■ catecholamines ■ norepinephrine transporter

Hemodynamic overload leads to increased cardiac mass and cardiomyocyte volume associated with characteristic changes in gene and protein expression. This initial compensatory hypertrophy is replaced by progressive structural/functional cardiac remodeling involving alterations in extracellular matrix composition, myocardial energetics, Ca\(^{2+}\) cycling, and myocyte viability.\(^1\)–\(^4\) Reactive oxygen species (ROS) contribute to each of these abnormalities,\(^5\)–\(^10\) but relevant sources of ROS remain to be fully defined. This is important, because recent studies suggest that specific targeting of ROS sources provides a more effective therapy for heart remodeling.\(^11\)

Monoamine oxidases (MAOs) are mitochondrial flavoenzymes which catalyze oxidative deamination of catecholamines (CAs) and biogenic amines such as serotonin. During this process, they generate hydrogen peroxide (H\(_2\)O\(_2\)) and thus can potentially be a source of oxidative stress in the heart, particularly under stress conditions. Yet, although the role of MAOs in terminating neurotransmitter signaling in the brain is well established,\(^12\) little is known about its modulation of cardiac morphology and function. MAOs exist in 2 isoforms, MAO-A and -B, with distinct substrate and inhibitor sensitivity.\(^12\) MAO-A, in particular, is present in the myocardium of several species from humans to rodents.\(^13\)–\(^15\)
### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>S-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
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<tr>
<td>S-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
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<tr>
<td>CA</td>
<td>catecholamine</td>
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<tr>
<td>CHF</td>
<td>congestive heart failure</td>
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<tr>
<td>DHPG</td>
<td>dihydroxyphenylglycol</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
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<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NET</td>
<td>norepinephrine transporter</td>
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<tr>
<td>PV</td>
<td>pressure-volume</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
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<td>WT</td>
<td>wild type</td>
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where it principally catabolizes serotonin, norepinephrine (NE) and epinephrine. All of these monoamine neurotransmitters have major functional implications in the heart, especially in the modulation of cardiac inotropy. A role for MAO-A and serotonin in ischemic/reperfused myocardium has been delineated showing that inhibiting MAO-A countermands oxidative stress, neutrophil accumulation and mitochondria-dependent cell death. Yet, the involvement of MAO-A and its impact on neurotransmitter availability in congestive heart failure (CHF) remains poorly defined, although one could speculate that this is the type of setting wherein MAO-A might be chiefly at play. CHF is accompanied by enhanced sympathetic tone, excess circulating CAs such as NE, and increased oxidative stress. In such situations, MAO-A may be upregulated, generating greater amounts of H₂O₂ thus exacerbating disease progression.

Here, we first determined whether cardiomyocyte (both neonatal and adult) exposure to NE is coupled to augmented MAO-A expression/activity, which in turn regulates a hypertrophic response linked to the generation of intracellular ROS. Adrenergic receptor–independent signaling was assessed using the MAO substrate tyramine. Second, we hypothesized that persistent hemodynamic stress imposed by in vivo pressure overload leads to excess CAs (and/or serotonin) presence at the myocyte surface resulting in increased sarcolemmal transport via extraneuronal monoamine transporter, thus enhancing substrate availability for intramyocyte MAO-A activity. To test this, we subjected mice to transverse aortic constriction (TAC), in the absence and presence of the highly selective MAO-A inhibitor clorgyline, and in mice expressing a dominant negative MAO-A (MAO-Aneo). Here, we show that NE triggers ROS and myocyte hypertrophy in part by a MAO-A dependent mechanism in vitro. In vivo, NE catabolism and ROS production are markedly upregulated in pressure overloaded hearts and both effects are ameliorated by inhibiting MAO-A activity to suppress cardiac decompensation with pressure overload.

### Methods

#### Animals

For pharmacological studies, male C57BL/6 mice (n=30, 9 to 11 weeks old) were used and clorgyline was administered using saline as vehicle (1 mg/kg per day, IP). For experiments using genetically modified mice, MAO-A+/− mice and their wild-type (WT) littermates in 129/Sv background were used (n=30). The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal experiments.

#### Statistics

All values are expressed as means±SEM. Comparison between groups was performed by 1-way or 2-way ANOVA, followed by a Tukey’s post hoc multiple comparison test. Comparisons between two groups were performed using nonpaired 2-tailed Student t test. A value of P<0.05 was considered significant.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Results

**Norepinephrine Triggers Cardiomyocyte Maladaptive Hypertrophy in a MAO-A–Dependent Manner**

We first tested whether externally applied NE (as when released from varicosities into the neuroeffector junctional areas) can enter myocytes, stimulating MAO-A activity and ROS–dependent signaling. Cultured rat neonatal cardiomyocytes were incubated with NE (10 μmol/L, 24 hours), which resulted in cell hypertrophy indexed by increased brain natriuretic peptide (BNP) gene expression and 1.3-fold increase in myocyte area (Figure 1A and 1B). This effect was accompanied by a rise in MAO-A gene expression and associated with a markedly increased production of mitochondrial ROS, measured with Mitotracker Red (Figure 1C and 1D). In these same experimental conditions, mitochondrial membrane potential was unchanged (Online Figure IV). Coincubation with the selective MAO-A inhibitor clorgyline (2 μmol/L) significantly blunted NE-induced ROS production and myocyte hypertrophy (Figure 1A, 1B, and 1D). Similar inhibition was obtained with 5 μmol/L clorgyline (40% to 50%, data not shown). To test the contribution of adrenergic receptor signaling, myocytes were incubated with tyramine, a MAO substrate that does not interact with adrenergic receptors yet is also transported into cells via the extraneuronal monoamine transporter. Myocytes treated with tyramine for 24 hours displayed a marked increase in mitochondrial oxidative stress (Figure 1D), and clorgyline prevented this change. Tyramine also increased myocyte area and BNP (Figure 1E and 1F), NFAT3 (nuclear factor of activated T cells), and NFAT4 (Figure 1G) gene expression; the latter are transcription factors implicated in maladaptive hypertrophy. Clorgyline inhibited tyramine-induced hypertrophy and suppressed increases in BNP and NFAT expression induced by either NE or tyramine.

We further tested these effects in adult cultured myocytes isolated from WT and MAO-Aneo mice, the latter expressing a dominant negative MAO-A, resulting in only 8% of residual MAO-A catalytic activity. WT myocytes challenged with NE displayed a marked increase in ROS production and this was significantly less in myocytes from MAO-Aneo mice.

### References

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
(Figure 2A). Likewise, increased cell area from NE exposure in WT myocytes was reduced in those from MAO-Aneo hearts (Figure 2B). These data further support the hypothesis that, in addition to adrenergic receptors, MAO-A derived ROS also contribute to NE-induced hypertrophy. The fact that in 2 different systems (neonatal rat and adult mouse myocytes) clorgyline or the genetic suppression of MAO-A activity produced a nearly equal reduction in ROS production and hypertrophy validates clorgyline as a specific MAO-A inhibitor.

**Norepinephrine Catabolism Is Increased in Failing (TAC) Hearts Because of Enhanced MAO-A Activity**

We next tested whether these findings pertained in vivo. Mice were subjected to TAC and MAO-A gene expression/activity was examined. As expected,19 TAC increased left ventricle (LV) mass/body weight ratio (+330% versus sham, \( P<0.001 \)), chamber dilation, and LV dysfunction, all features of adverse remodeling (Table 1). In these hearts, MAO-A gene expression was 3-fold higher (Figure 3A, top). To test...
for MAO-A activity, we initially performed in vitro activity assays using $^{14}$C-labeled serotonin as a substrate. No significant differences were observed after 6 weeks of TAC versus sham-controls (Figure 3A, bottom). MAO activity was also indexed by H$_2$O$_2$ production in tissue homogenates in the presence of substrates. Again, no difference was evident (data not shown). To test whether MAO-A activity was upregulated in vivo as a result of increased substrate availability, we measured cardiac NE and serotonin levels in LV specimens of sham and TAC mice. We also assessed the absolute content of dihydroxyphenylglycol (DHPG) and 5-hydroxyindoleacetic acid (5-HIAA) that are the primary catabolic products of NE and serotonin, respectively, resulting from reactions catalyzed by MAO-A.$^{26}$ Absolute cardiac amount of DHPG was significantly elevated in LV tissue extracts of TAC mice, with a consistent marked

Table 1. Time-Dependent Changes in Cardiac Morphology and In Vivo Ventricular Function Induced by TAC in Control and Clorgyline-Treated Mice

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=5)</th>
<th>Sham + Clo (n=5)</th>
<th>T3w (n=8)</th>
<th>T3w + Clo (n=8)</th>
<th>T6w (n=8)</th>
<th>T6w + Clo (n=8)</th>
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</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>27.3±0.7</td>
<td>25.7±1.4</td>
<td>25.3±0.6</td>
<td>25.9±0.6</td>
<td>26.6±0.7</td>
<td>27.3±0.3</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>683±21</td>
<td>690±8</td>
<td>674±11</td>
<td>701±11</td>
<td>628±24</td>
<td>665±14</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.9±0.02</td>
<td>0.8±0.02</td>
<td>1.3±0.04*</td>
<td>1.2±0.05*</td>
<td>1.2±0.04*</td>
<td>1.3±0.06*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.2±0.07</td>
<td>3.2±0.09</td>
<td>2.9±0.2</td>
<td>3±0.1</td>
<td>4±0.2*</td>
<td>3.1±0.08†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.3±0.04</td>
<td>1.2±0.06</td>
<td>1.7±0.2</td>
<td>1.3±0.09</td>
<td>2.9±0.4*</td>
<td>1.2±0.05§</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.8±0.02</td>
<td>0.8±0.03</td>
<td>1.3±0.05*</td>
<td>1.2±0.04*</td>
<td>1.1±0.05*</td>
<td>1.2±0.05*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>60.4±0.9</td>
<td>62.7±1.1</td>
<td>43.2±3.5*</td>
<td>56.5±1.9†</td>
<td>30.6±5.3*</td>
<td>61.1±1‡</td>
</tr>
<tr>
<td>EF (%)</td>
<td>93.7±0.4</td>
<td>94.7±0.5</td>
<td>80.2±3.6*</td>
<td>91.4±1.1†</td>
<td>62.4±7.9*</td>
<td>94.0±5§</td>
</tr>
<tr>
<td>LV mass/BW</td>
<td>4.3±0.1</td>
<td>4.5±0.05</td>
<td>6.9±0.5*</td>
<td>6.5±0.3*</td>
<td>9.3±0.5*</td>
<td>6.9±0.4‡</td>
</tr>
</tbody>
</table>

BW indicates body weight; Clo, clorgyline; EF, ejection fraction; FS, fractional shortening; IVS, interventricular septum; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVPW, left ventricular posterior wall; T3w, TAC for 3 weeks; T6w, TAC for 6 weeks. *P<0.05 vs respective sham; †P<0.001 vs TAC 3 weeks; ‡P<0.05 vs TAC 6 weeks.

Figure 3. MAO-A activity and neurotransmitter catabolism is enhanced in pressure overloaded hearts. A, MAO-A gene expression normalized to GAPDH (top) and in vitro MAO-A activity (bottom) in hearts from sham-operated and 6-weeks TAC mice. B, NE and DHPG content and DHPG/NE ratio in sham and TAC hearts treated without or with clorgyline. C, 5-HT and 5-HIAA content and 5-HIAA/5-HT ratio in the same groups. D, Western blot for NET and quantification of bands intensity (results normalized to GAPDH expression). T6w indicates TAC 6 weeks; Clo, clorgyline. *P<0.05 vs respective sham; **P<0.005 vs respective sham; †P<0.05 vs T6w; ††P<0.005 vs T6w.
decrease in cardiac NE content (Figure 3B). When cardiac DHPG content was normalized to intracardiac NE levels, the DHPG/NE ratio was 4-fold higher in TAC hearts versus shams (Figure 3B). Thus, in TAC hearts NE catabolism is increased. Evidence showing that MAO-A inhibition by clorgyline fully reverted the rise in DHPG and DHPG/NE ratio while rescuing the intracardiac content of NE available for release (Figure 3B) supports the specific involvement of MAO-A in this setting.

We also determined whether serotonin, another elective MAO-A substrate, is equally important in this CHF model. Serotonin levels were unaltered by TAC, and rose only after clorgyline treatment, consistent with MAO-A inhibition (Figure 3C). In stark contrast to DHPG, serotonin catabolism measured by 5-HIAA and 5-HIAA/serotonin ratio was not altered in TAC hearts compared to shams. However, clorgyline significantly reduced 5-HIAA/serotonin ratio after TAC. Together, these data show NE to be the preferred substrate fueling MAO-A activity in this model.

Intriguingly, the protein expression of the neuronal NE transporter (NET) declined with TAC, likely reducing neuronal NE reuptake. Clorgyline treated TAC mice had normal NET expression (Figure 3D) and higher neuronal NE levels.

MAO-A Inhibition Prevents LV Dilation and Dysfunction in TAC Hearts

We next examined whether MAO-A inhibition was also effective in blunting maladaptive responses to sustained pressure overload in vivo. After 3 weeks of TAC, both saline- and clorgyline-treated hearts developed increased wall thickness, but the latter hearts also had preserved LV function: fractional shortening and ejection fraction values were indeed similar to those reported for sham-controls (Table 1). This beneficial impact on cardiac structure and function became even more pronounced 6 weeks after TAC. Heart weight to body weight ratio was significantly reduced in the clorgyline-group (Figure 4B), and end-diastolic and end-systolic dimensions were less and not dissimilar from values seen in sham-operated mice (Table 1 and Figure 1A). Cardiac function was also fully preserved. Pressure–volume (PV) analysis was also performed to assess if clorgyline had any effects on basal cardiac function over the same time-course of treatment. We found no differences (Online Figure I).

Maladaptive hypertrophy is typically paralleled by fetal gene reprogramming with increased cardiac expression of atrial natriuretic peptide, BNP, and β-MHC. Therefore, we tested whether clorgyline treatment restored the adult phenotype expression. LV mRNA expression for atrial natriuretic peptide, BNP and β-MHC increased markedly in TAC hearts (Figure 4C), and clorgyline treatment lowered expression of all genes by 2-fold. Thus, MAO-A inhibition prevents both adverse cardiac structural and gene reprogramming in pressure-overloaded hearts.

Oxidative Stress, Matrix Metalloproteinase Activation, and Apoptosis Are Blunted in Clorgyline-Treated TAC Hearts

Altered cardiac tissue redox balance is a hallmark of CHF, and MAO-A is a recognized source of ROS in ischemia/reperfusion injury. Yet, whether MAO-A upregulation contributes to CHF pathophysiology in part via enhanced oxidative stress is unknown. Consistent with previous reports, ROS were markedly elevated in myocardium of 6-weeks TAC mice detected by dihydroethidium staining (Figure 5A). This burden was significantly reduced by clorgyline. Results were further confirmed by measuring tissue malondialdehyde, an index of lipid peroxidation. Malondialdehyde amount in TAC hearts was significantly lower after MAO-A inhibition (Figure 5B).

Because ROS are among the activators of gelatinases such as matrix metalloproteinase (MMP)-2 and MMP-9 and MMPs are upregulated in pressure-overloaded hearts, we tested whether this was also suppressed by clorgyline. Activities of both MMP-2 and MMP-9 were upregulated in TAC
hearts, and clorgyline reduced this (Figure 5C). We also tested whether myocardial apoptosis was involved in TAC-induced CHF and blunted by clorgyline. Cleaved (activated) caspase-3 (Figure 5D) and the number of apoptotic cells measured by TUNEL assay (Figure 5E) markedly rose after 6 weeks of TAC. Clorgyline significantly blunted these adverse phenomena.

**MAO-Aneo Mice Display Improved LV Function, No Chamber Dilation, and Reduced Levels of Fibrosis After TAC**

To further establish the specific involvement of MAO-A in TAC-induced cardiac remodeling, we studied mice lacking enzyme activity because of expression of a dominant negative MAO-A (MAO-Aneo). These mice display almost null MAO-A activity (Figure 6A) but have preserved levels and activity of MAO-B (Online Figure II). The cardiac phenotype of these mice had yet to be fully characterized, thus load-independent LV function and hemodynamics were examined using in vivo PV relationships. WT and MAO-Aneo mice were somewhat different at baseline, with LV systolic pressure, dP/dt\(_{\text{max}}\), and dP/dt\(_{\text{min}}\) all lower in MAO-Aneo compared to WT littermates (Table 2). Contractile function assessed by preload recruitable stroke work index was also lower in MAO-Aneo mice, so these differences were potentially related to loading changes. However, chamber volume and ejection fraction were similar between the two strains. When WT and MAO-Aneo mice were subjected to 9 week of TAC, WT hearts had a greater dilation, with a rightward shift in the PV loop (Figure 6A and 6B), whereas LV function became impaired. In contrast, MAO-Aneo mice showed a slight leftward shift of the PV relations, with preserved (basal) cardiac volumes, and maintained cardiac function. Consistent with the data obtained in C57BL/6 mice, 129/Sv WT mice also displayed reduced NET protein abundance after CHF. In stark contrast, NET levels in MAO-Aneo mice were similar to those reported for sham operated mice (Figure 6C). Compared to WT, MAO-Aneo mice subjected to TAC also had less interstitial fibrosis (Figure 6D). Thus, genetic inhibition of MAO-A activity also helped ameliorate structural-functional consequences of chronic pressure overload.

**Discussion**

Changes in cardiac tissue redox balance participate in myocyte hypertrophy and failure, influencing extracellular matrix remodeling, Ca\(^{2+}\) handling, and metabolic substrate.\(^6,10,28\)
Clarification of the important sources of ROS therefore has pathogenetic and therapeutic relevance. In the cytosol, NADPH oxidase, xanthine oxidase, and uncoupled nitric oxide synthase are recognized ROS sources. Mitochondria are another major source, largely from the respiratory chain and p66Shc, and are known to contribute to ischemia/reperfusion injury. Within mitochondria, the flavoenzyme MAO-A, located in the outer membrane of the organelle, is a major ROS generator. MAO-A activity is implicated in serotonin-induced myocyte apoptosis and ischemia/reperfusion injury via a ROS-dependent process involving sphingosine kinase inhibition and accumulation of ceramide. MAO-derived ROS also appear relevant to serotonin-induced myocyte hypertrophy in vitro.

In this study, we showed that in addition to serotonin, NE catabolism by MAO-A plays a prominent role in hypertrophy in vitro and in its progression toward heart failure in vivo. Catecholamines, and NE in particular, are known to contribute to cardiac disease and couple to ROS signaling. Enhanced CA synthesis and release may provide help for adaptation to increased workload; however, because NE is a high affinity substrate for MAO-A, this can also serve as a major factor for increased ROS, as supported by our in vitro myocyte data. Clorgyline partly prevented this change, whereas the remaining prooxidant/hypertrophic effects were likely attributable to adrenergic receptor–coupled mechanisms. Furthermore, NE metabolism by MAO-A increased in TAC mice, which was also associated with exacerbated oxidative stress, chamber dilation, and reduced systolic function. Pharmacological inhibition of MAO-A suppressed these changes.

Impairment of NE neuronal reuptake and concomitant downregulation of the β-adrenergic system are well documented in human and experimental CHF, contributing to the loss of systolic performance in this syndrome. In normal hearts, 92% of the NE released by sympathetic nerves is recaptured by NET, 4% is removed by extraneuronal uptake, and the remaining 4% enters the circulation. However, NET function declines in CHF, resulting in NE spillover and extraneuronal uptake which almost doubles in CHF patients. Also, the balance between vesicular NE sequestration (which represents the “measurable” NE pool) and leakage in the intracardiac sympathetic efferent fibers may be altered to favor extraneuronal uptake, providing more substrate for MAO-A. The present results showing reduced NET expression in TAC hearts support this hypothesis. Increased ROS
Nine Weeks of TAC increases the number of NET recognition sites. This finding finds that systemic administration of MAO inhibitors ablation of MAO-A catalytic activity restored NET expression back to control levels in TAC hearts, consistent with present findings, other factors might also contribute, such as the severity of the pressure overload. In the prior study, even the WT mice showed little dilation and preserved function. The authors also used WT as opposed to littermate controls (the latter employed in the present study). The C57BL/6 strain develops more severe responses to TAC, and the benefits of clorgyline, which avoids potential adaptive changes in the MAO-A gene deletion mouse models, supports the opposite response. Although our control mice for the MAO-Aneo studies (129/Sv background) developed less hypertrophy (consistent with prior reports), there was still substantial dilation that was ameliorated in mice lacking active MAO-A. Other potential contributors to this discrepancy are difference in functional assessment, with anesthesia-induced cardiodepression in the earlier study, versus conscious data in the present experiments. MAO-Aneo mice also had basal elevated intracardiac NE content. It remains to be determined whether myocyte-specific gene deletion would yield the same results. In the earlier study of Lairez et al, cardiac levels of CAs were not determined whereas serotonin levels were found to be increased at baseline in MAO-A−/− mice, consistent with present findings, and did not change when LV remodeling was already established. The latter is also consistent with our study in showing no alteration of serotonin content in TAC-induced CHF. As expected, in both studies MAO-A inhibition increased serotonin levels in TAC hearts. Thus, considering that increased cardiac serotonin content is concomitant with improved LV function and absence of remodeling after TAC, an actual beneficial effect of serotonin cannot be excluded. It is plausible that serotonin may sustain cardiac contractility during late stage CHF, particularly if a deficit in NE availability persists. In the end, this effect would provide an additional explanation for the beneficial action of MAO-A inhibition.

There are some limitations to this study. We did not test the role of MAO-B and catechol-O-methyl transferase (COMT), both additional monoamine catabolic enzymes. However, prior studies in mice and humans have not shown a major role for MAO-B in NE catabolism, and COMT gene expression did not change in our TAC model (data not shown). Future studies with pharmacological and genetic ablation of these enzymes will be required to definitively examine their role. The mechanistic intricacies by which MAO-A inhibition preserves NET expression, and likely function (given clorgyline effects on NE content in shams and TAC hearts), awaits further investigation, but a possible major involvement of oxidative/nitrosative stress seems plausible.

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### Table 2. Morphological and Functional Changes via PV Relationships in WT and MAO-A^neo^ Mice at Baseline and After Nine Weeks of TAC

<table>
<thead>
<tr>
<th>Parameter and Genotype</th>
<th>Sham</th>
<th>TAC 9 Weeks</th>
</tr>
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<tr>
<td>Heart rate (bpm)</td>
<td></td>
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<tr>
<td>WT</td>
<td>537±10</td>
<td>503±23</td>
</tr>
<tr>
<td>MAO-A^neo^</td>
<td>515±28</td>
<td>501±19</td>
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<tr>
<td>Peak LVPs (mm Hg)</td>
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<td></td>
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<tr>
<td>WT</td>
<td>119±6</td>
<td>162±14†</td>
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<tr>
<td>MAO-A^neo^</td>
<td>97±6*</td>
<td>137±17†</td>
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<tr>
<td>LVPsia (mm Hg)</td>
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<tr>
<td>WT</td>
<td>1±0.3</td>
<td>2±1</td>
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<tr>
<td>MAO-A^neo^</td>
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<td>2±0.7</td>
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<tr>
<td>LWes (μL)</td>
<td></td>
<td></td>
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<tr>
<td>WT</td>
<td>4.5±1</td>
<td>16.6±4.5‡</td>
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<tr>
<td>MAO-A^neo^</td>
<td>5.7±2.4</td>
<td>5.5±1.4§</td>
</tr>
<tr>
<td>LWed (μL)</td>
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<tr>
<td>WT</td>
<td>27.1±2.5</td>
<td>39.4±3.1†</td>
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<tr>
<td>MAO-A^neo^</td>
<td>28.1±5</td>
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<tr>
<td>EF (%)</td>
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<td>83.8±1.9</td>
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<td>dP/dt\text{max} (mm Hg/sec)</td>
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<td>MAO-A^neo^</td>
<td>−8013±948*</td>
<td>−9663±1192</td>
</tr>
<tr>
<td>τ (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5.6±0.2</td>
<td>5.7±0.1</td>
</tr>
<tr>
<td>MAO-A^neo^</td>
<td>5.9±0.1</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>PRSW (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>129.6±8.2</td>
<td>132.3±11</td>
</tr>
<tr>
<td>MAO-A^neo^</td>
<td>95.3±11.1*</td>
<td>126.2±20.9</td>
</tr>
</tbody>
</table>

* indicates relaxation constant; EF, ejection fraction; LVPSia, left ventricular diastolic pressure; LVPSed, left ventricular systolic pressure; LVWes, end-systolic left ventricular volume; LVWes, end-systolic left ventricular volume; PRSW, preload recruitable stroke work. †P<0.05 vs WT sham; ‡P<0.05 TAC vs sham; ††P<0.01 vs WT sham; §§P<0.05 vs WT TAC; comparison performed by t test (n=5 each group).

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generation could stem from intramyocyte and/or intraneuronal MAO-A activity as clorgyline inhibits both. The former is supported by the present findings (e.g., Figures 1 and 5A and 5B), whereas proof of the latter would require studies in isolated sympathetic efferent fibers and/or nerve-muscle preparations. Our data also showed that clorgyline or genetic ablation of MAO-A catalytic activity restored NET expression back to control levels in TAC hearts, consistent with findings that systemic administration of MAO inhibitors increases the number of NET recognition sites. This finding hints at the possibility that MAO-A inhibition may also benefit both NE reuptake and intraneuronal CAs recycle for rerelease, thereby reducing requirements for transmitter neosynthesis.
nally, any posttranscriptional and/or posttranslational regulation of MAO-A is currently under investigation.

In conclusion, the present data support MAO-A as an important source of ROS that contributes to maladaptive remodeling and myocardial dysfunction in hearts subjected to hemodynamic stress. The latter likely results in NE-mediated MAO-A activation attributable to depressed neuronal uptake. Whether inhibited ROS production and improved NE cycling/availability are the only keys to interpret the beneficial impact of MAO-A inhibition in pressure overloaded hearts needs further investigation. However, present findings add MAO-dependent signaling as a cause of stress-induced maladaptive hypertrophy and pump failure. The data also suggest that MAO-A inhibitors may prove useful in other models of cardiac failure. In the past, nonselective MAO inhibition was associated with the so-called “cheese reaction,” consisting of severe hypertensive crises following the ingestion of food rich in tyramine. However, the generation of new MAO-A inhibitors lacking this limiting side effect makes even more attractive the idea of advancing such therapy for clinical use in CHF patients.

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We gratefully acknowledge Dr Graeme Eisenhofer for insightful suggestions and critical revision of the manuscript and Raymond Johnson at Vanderbilt Neuroscience Core for invaluable help with high-performance liquid chromatography assays. We also thank the Ross confocal facility (NIH/R24DK064388, The Hopkins Basic Research Digestive Disease Development Core Center) for confocal microscope access and John Gibas for technical assistance.

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Disclosures

None.

References


Monoamine Oxidase A–Mediated Enhanced Catabolism of Norepinephrine Contributes to Adverse Remodeling and Pump Failure in Hearts With Pressure Overload


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Supplemental Material.

Supplemental methods.

Generation of MAO-A\textsuperscript{neo} mice. MAO-A\textsuperscript{neo} mouse was generated by alteration of a 9.0kb Bam HI fragment of MAO-A gene containing exon 10 to exon 13 of mouse MAO-A gene. A 0.28kb Smal-EcoRI loxP fragment was blunt and ligated into a unique SphI site in intron 11 of MAO-A and a 1.6kb EcoRI-BgIll floxed neomycin cassette was directionally cloned into in the same sites within the intron 12 of MAO-A gene (Online Figure V). MAO-A\textsuperscript{neo} mice do not have MAO-A activity. The full MAO-A activity was restored by crossing MAO-A\textsuperscript{neo} mice with CMV-cre mice and selectively deleting the 1.6 kb floxed neomycin insert in intron 12.

Genotyping of MAO-A\textsuperscript{neo} mice. Primers flanking the loxP insert in intron 11 were designed for genotyping: forward primer (F): 5'-CCTCTCTTCCAAGTATTAGG-3'; reverse primer (R): 5'-GGAAAAGAGGGAGGAGTAAG-3'. Tail clipped genomic DNA was used as template, DNA fragment was amplified by a 4 min hot start at 94°C followed by 35 cycles of 30 sec at 94°C, 40 sec at the optimal annealing temperature (50°C), 45 sec at 72°C for elongation and a final 5 min extension at 72°C. The PCR product size is 300 bp and 500 bp for WT and MAO-A\textsuperscript{neo} mouse, respectively.

TAC procedure. TAC was performed following a previously reported protocol\textsuperscript{1,2}. After induction of anesthesia and intubation, mice were placed on a volume ventilator (120 breaths/min, 1.2 ml/g/min) and anesthesia was maintained with 5% isoflurane. The aortic arch was isolated and tied against a 27-gauge needle, resulting in a 65-70% constriction after the removal of the needle. The chest and skin were closed and animals extubated and allowed to fully recover. Sham-operated mice underwent the same operation except that after the aortic arch was isolated, there was no ligature placed.

Echocardiography. In vivo cardiac morphology and function were assessed by serial M-mode echocardiography (Acuson Sequoia C256, 13 MHz transducer, Siemens, PA) performed in conscious mice. LV end-systolic and end-diastolic dimensions were averaged from 3-5 beats. LV percent FS, EF and LV mass were calculated as described previously\textsuperscript{3}. Thickness of posterior free wall and interventricular septum were averaged.

In Vivo Pressure-Volume Loop Studies. In vivo cardiac function was assessed by pressure-volume catheter in anesthetized mice employing a four-electrode pressure-volume catheter (model SPR-839, Millar Instruments, TX, USA) as previously described\textsuperscript{2,4,5}. Anesthesia was maintained with i.p. etomidate (250 µg), urethane (30mg) and morphine (15 µg). Mouse was placed in supine position on a thermoregulated surgical table maintained at 37°C. Ventilation via endotracheal tube was maintained with 100% oxygen using a custom-designed constant flow ventilator delivering a tidal volume of 6.7 µl/g at 120 breaths/min. An internal jugular venous line was placed to provide a fluid and drug delivery port. The thorax was opened and a miniature PV catheter inserted into the left ventricle via the apex for continuous LV pressure-volume data. Calibration of the signal was performed using injection of hypertonic saline and direct measurement of cardiac output via an aortic flow probe (Transonic Instruments) placed around thoracic descending aorta or a pulse wave Doppler signal at the aortic outflow (Indus Instruments, Houston, TX). Pressure-volume loop analysis was made using a custom analysis program (WinPVAN 3.3).

Neonatal rat cardiomyocytes studies. Myocytes were isolated from 1-2 days old Sprague Dawley rats, as previously described\textsuperscript{6}. After 2 days of culture, myocytes were pretreated with
clorgyline for 1 hour and then subjected to stimulation with norepinephrine or tyramine (10 and 20 μmol/L respectively). After 24 hours, cells were harvested for RNA isolation using Trizol (Invitrogen). For cell area measurements, cells were fixed, incubated with α-actinin primary antibody (1:500 dilution, Sigma) and fluorescent secondary antibody (Molecular probes) and visualized by confocal microscopy (Zeiss, LSM 510 Meta). Approximately 150-200 cells were analyzed per condition in each experiment. All the experiments were repeated at least four times. For mitochondrial ROS production measurements, the reduced form of Mitotracker Red (Mitotracker Red CM-H2XRos, Molecular probes) was used. Cells were loaded with 100 nmol/L Mitotracker Red CM-H2XRos for 30 minutes at 37˚C, washed and then incubated with NE or vehicle for 2 hours. After this incubation time, cells were analyzed and images acquired by confocal microscopy. Images were analyzed and fluorescence intensities determined using computer-assisted image analysis systems (ImageJ, NIH). For the mitochondrial membrane potential measurements, cells were loaded with 20 nmol/L tetramethylrodamine (TMRM) for 30 minutes and then treated with NE for 2 hours. Images were acquired and analyzed using computer-assisted image analysis systems (ImageJ, NIH).

**Measurement of MAO-A activity.** MAO-A activity in the heart (expressed as nmol per 20 min per milligram of protein) was determined by use of 14C labeled serotonin (5-HT) as a substrate. Alternatively, MAO-A activity was determined by Amplex Red assay (Invitrogen), measuring hydrogen peroxide formation.

**Catecholamine measurement.** In order to remove any contaminating blood from the LV specimens, after the excision the hearts were retrogradely perfused in a Langerdorff apparatus for 10 min using K-H solution. Then, LV specimens were dissected and homogenized in 0.4 mol/L perchloric acid containing 0.5 mmol/L EDTA, centrifuged at 3 000 rpm for 5 minutes and the supernatant stored for alumina extraction. CA and 5-HT were determined by HPLC as described previously. CA/5-HT content is expressed as a function of protein content.

**Histology.** Hearts were fixed in 10% formalin overnight, embedded in paraffin, sectioned at 5 μm thickness and stained using H&E and Masson’s trichrome. Photomicrographs of the sections were evaluated for interstitial collagen fractions using computer-assisted image analysis systems (ImageJ, NIH).

**Apoptosis detection by TUNEL staining.** The assay was performed using a commercially available kit according to manufacturer’s instructions (CardioTACs). TUNEL-positive nuclei were visualized using a light microscope equipped with a camera. The percentage of TUNEL-positive cardiomyocytes is expressed as a percentage of total nuclei.

**Measurement of oxidative stress.** Oxidative stress was determined by MDA formation measurement and DHE staining. MDA, an end product of lipid peroxidation, was determined spectrophotometrically by measurement of 2-thiobarbituric acid reactive substances (TBARS), as described previously. For DHE staining, 10 μm thick cryosections were incubated with 5 μmol/L DHE (Sigma) for 30 minutes at 37˚C, washed twice with PBS, mounted and visualized using a confocal microscope (Zeiss, LSM 510 Meta).

**MMP activity measurement.** In vitro gelatin lysis by MMP-2 and MMP-9 was assessed by zymography as previously described. Briefly, protein concentration was determined and equal amounts of lysed tissue samples were loaded on a 10% gelatin gels (Invitrogen Corp.). After electrophoresis, gels were washed twice with renaturing buffer at room temperature followed by developing buffer (Invitrogen Corp.) and then stained to visualize lytic bands (SimplyBlue;
Invitrogen Corp.). Lytic bands corresponding to active forms of MMP-2 and MMP-9 were quantified using the BioRad analysis software.

**Western Blot and real time PCR.** Western blot was performed as previously described\(^6\). RNA was isolated, purified and reverse transcribed using commercially available kits (Qiagen, Invitrogen). cDNA was subjected to PCR amplification (Abi Prism 7000 Detection System, Applied Biosystems) using SYBR Green dye (Applied Biosystems). Primer sets for the specific target genes were designed to span one or more introns (IDT Technologies).

**Adult mouse ventricular myocytes (AMVM) isolation and culture.** AMVMs were isolated from the hearts of adult (12 weeks) male MAO-A\(^{neo}\) mice and their WT littermates as described previously\(^11\). Cells were plated at a nonconfluent density of 25 000 rod shaped cells/ml on plastic culture dishes or glass coverslips precoated with laminin (20 µg/ml) and kept at 37°C in the culture medium (DMEM, Joklik modified MEM, NaHCO\(_3\) 2 g/l, BSA 5 g/l, L-carnitine 1.5 mmol/L, creatine 5 mmol/L, taurine 7.5 mmol/L, ITS 1%, penicillin 100 IU/mL, streptomycin 10 µg/mL) for 1 hour before being used for the experiments. For mitochondrial ROS production measurements, cells were loaded with 100 nmol/L Mitotracker Red CM-H\(_2\)XRos for 30 minutes at 37°C, washed and then incubated with NE (10 µmol/L) or vehicle for 2 hours. After this incubation time, cells were analyzed and images acquired by confocal microscopy. Images were analyzed and fluorescence intensities determined using computer-assisted image analysis systems (ImageJ, NIH). For cell area measurements, cells were incubated with NE (1 µmol/L) for 24 hours and visualized by confocal microscopy (Zeiss, LSM 510 Meta). Approximately 150-200 cells were analyzed per condition in each experiment and all the experiments were repeated at least three times.


Online Figures

**Online Figure I.** Hemodynamic assessment of control (vehicle) and clorgyline- treated mice. A: Representative PV loops at baseline and after IVC occlusion from 6 weeks vehicle and clorgyline-treated mice. B: Heart rate (HR), stroke volume (SV), dp/dt$_{max}$ and preload recruitable stroke work (PRSW) in vehicle and clorgyline treated mice. (n=5 each)
Online Figure II. MAO-B expression and activity in MAO-A\textsuperscript{neo} mice. A: MAO-B mRNA levels in hearts from WT and MAO-A\textsuperscript{neo} mice. B: MAO-B activity in hearts from WT and MAO-A\textsuperscript{neo} mice. (n=5 each)
Online Figure III. NE (panel A) and serotonin (panel B) levels in hearts from WT and MAO-A<sup>neo</sup> mice at baseline and after 9 weeks of TAC. §p<0.05 vs WT sham, #p<0.05 vs WT T9w. (n=5 each)
**Online Figure IV.** Mitochondrial membrane potential determined by TMRM after 2 hours of incubation with 10 μmol/L NE in the absence or in the presence of clorgyline (Clo).
Online Figure V. Schematic representation of MAO-A<sub>neo</sub> mice generation. The insertion of 0.28kb loxP and 1.6 kb loxP-pgk1-Neo-loxP cassette into intron 11 and intron 12 of MAO A gene. A loxP (0.28kb SmaI-EcoRI) fragment was blunt end ligated into a unique Sp site in intron 11. Another 1.6kb E-Bg floxed neo cassette was directionally cloned into the E-Bg site in intron 12 of MAO-A gene. (Sp: SphI; E; EcoRI; Bg: Bgl II restriction enzyme).