Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection

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Aims We have shown previously that non-toxic inhibition of the ubiquitin–proteasome system upregulates antioxidative defence mechanisms and protects endothelial cells from oxidative stress. Here, we have addressed the question whether the induction of antioxidative enzymes contributes to cardioprotection by non-toxic proteasome inhibition.

Methods and results Treatment with 0.5 μmol/L MG132 for 48 h proved to be non-toxic and protected neonatal rat cardiac myocytes against H_2O_2-mediated oxidative stress in lactate dehydrogenase assays. This correlated with reduced levels of intracellular reactive oxygen species as determined by loading myocytes with dichlorofluorescein. Immunoblots showed significant upregulation of superoxide dismutase 1 (SOD1), haem oxygenase 1, and catalase upon proteasome inhibition. Luciferase assays using a reporter driven by the SOD1 promoter revealed proteasome inhibitor-mediated induction of luciferase activity. Deletion and mutation analyses identified an antioxidant response element (ARE) in the SOD1 promoter to be not only essential but also sufficient for transcriptional upregulation by proteasome inhibition. An essential role for the antioxidative transcription factor NF-E2-related factor 2 (Nrf2)—which was stabilized by proteasome inhibition—in ARE-mediated transcriptional activation was revealed in cardiac myocytes from Nrf2 wild-type and knockout mice: proteasome inhibition upregulated antioxidative enzymes and conferred protection against H_2O_2-mediated oxidative stress in Nrf2 wild-type cells. In contrast, the induction of antioxidative enzymes and cytoprotection were completely abolished in cardiac myocytes from Nrf2 knockout mice.

Conclusion Non-toxic proteasome inhibition upregulates antioxidative enzymes via an Nrf2-dependent transcriptional activation of AREs and confers cardioprotection.

1. Introduction

Oxidative stress plays an important role in the pathophysiology of various cardiovascular diseases, e.g. endothelial dysfunction, atherosclerosis, and ischaemia–reperfusion injury. Supraphysiological levels of reactive oxygen species (ROS)—such as hydrogen peroxide, superoxide anions, and hydroxyl radicals—severely damage proteins, DNA, and lipids, which results in further tissue damage and organ dysfunction. Several studies investigating the effects of different defence mechanisms against ROS, e.g. treatment with antioxidants,[1,2] chelators,[3] or antioxidative enzymes,[4–7] reported enhanced survival of cardiac myocytes, reduced infarction size, and reduction of myocardial dysfunction in models of ischaemia–reperfusion injury. These observations suggest beneficial effects of therapeutic strategies aiming at the reduction of ROS-mediated cell damage.

The ubiquitin–proteasome system is the major protein degradation pathway of eukaryotic cells. It is essential for the regulated degradation of proteins involved in protein turnover and quality control,[8] cell cycle and apoptosis,[9,10] as well as transcription and cell signalling.[11–13] Hence, proteasome inhibition has been established as a powerful and promising therapeutic strategy for the treatment of various human diseases. Bortezomib (*velcade*®) was the first proteasome inhibitor to be approved by the FDA in 2003. Other inhibitors are currently being tested in clinical trials of malignant diseases.[14] Proteasome inhibitors have also been proposed as anti-inflammatory remedies because of their ability to block the activation of the central inflammatory transcription factor nuclear factor kappa B (NFκB).[15]

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We have shown recently that protection of endothelial cells against oxidative stress by proteasome inhibition is associated with the upregulation of several antioxidative enzymes. In particular, glutathione peroxidase 3, haem oxygenase 1 (HO1), superoxide dismutase 1 (SOD1), and several glutathione *S*-transferases were upregulated in a concerted manner between 2- and 20-fold.[16] Our results are corroborated by a current study which shows that proteasome inhibition confers neuroprotection and induces γ-glutamylcysteine synthetase—a rate-limiting enzyme in glutathione synthesis.[17] These data suggest that proteasome inhibition represents a promising strategy to confer protection against oxidative stress in different cell types. Here, we have analysed the protective effects of proteasome inhibition on cardiac myocytes and the underlying mechanisms of proteasome inhibitor-induced upregulation of antioxidative enzymes in particular.

2. Methods

2.1 Primary cardiac myocyte cell culture

The investigation conforms with local university guidelines as well as with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval was also granted by the local office for animal protection and research (Landesamt für Gesundheit und Soziales Berlin, approval numbers T0275/07 and T0408/98). Cardiac myocytes were prepared from neonatal Wistar rats or C57BL6J NF-E2-related factor 2 (Nrf2) wild-type and knockout mice as described previously and plated in six-well plates (10^6 cells per well, ca. 10^3 cells/mm^2).[18,19] Neonatal rat cardiac myocytes (NRCM) were kept in medium M199 containing 5% newborn calf serum (Invitrogen, Karlsruhe, Germany); neonatal murine cardiac myocytes (NMCM) were cultured in a mixture of DMEM and M199 (DMEM:M199 3:1, 10% horse serum, 5% newborn calf serum, 100 U/mL penicillin–streptomycin, 1 mmol/L HEPES, pH 7.4). At the time point of stimulation, cells had reached confluency of ~70–80%.

2.2 Model for oxidative stress

Forty-eight hours after plating, NRCM were treated in the presence of serum with 0.5 μmol/L of the cell permeable, reversible, peptide aldehyde proteasome inhibitor MG132 (Z-Leu-Leu-Leu-CHO, $K_i = 4$ nM, Merck, Schwalbach, Germany)[20] or solvent (0.1% DMSO) for 48 h. This dose proved to be non-toxic but exerted significant protective effects. NMCM were treated with 0.3 μmol/L MG132 or solvent (0.1% DMSO) for 48 h 4 days after plating. For the induction of oxidative stress, cells were washed with PBS, incubated with hydrogen peroxide for 30 min in serum-free medium and further kept in serum-free standard medium for up to 24 h. NRCM were incubated with 30 μmol/L H_2O_2, NMCM with 100 μmol/L H_2O_2.

2.3 Quantification of cell damage

Cell damage was determined by measuring the release of lactate dehydrogenase (LDH) into the cell culture medium. The release was quantified using the CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega, Mannheim, Germany) according to the manufacturer's protocol. In NRCM, cellular damage was assessed 48 h after treatment with MG132, as well as 4 and 24 h after the induction of oxidative stress by incubation with H_2O_2. In NMCM, the damage was measured 24 h after the induction of oxidative stress.

2.4 Measurement of proteasome activity

Since MG132 mainly inhibits the chymotrypsin (ChT)-like activity,[16] we determined the proteasome activity by quantifying the hydrolysis of SLLVY-AMC—a fluorogenic substrate for the ChT-like activity—in a fluorescence reader (Gemini EM, Molecular Devices, Munich, Germany) as described previously.[21] Briefly, NRCM were treated with 0.5 μmol/L MG132 or 0.1% DMSO for 24 and 48 h. NRC were then lysed in aqua dest with repeated cycles of thawing and freezing in liquid nitrogen. After centrifugation, protein concentrations were determined using the Bradford method (Pierce, Rockford, IL, USA). Lysates were incubated for 30 min at 37°C in incubation buffer containing 0.2 mmol/L SLLVY-AMC and an ATP-regenerating system [225 mmol/L Tris–HCl, pH 8.2, 45 mmol/L KCl, 7.5 mmol/L Mg(CH$_3$COO)$_2$, 7.5 mmol/L MgCl$_2$, 1.1 mmol/L dithiothreitol, 6 mmol/L ATP, 5 mmol/L phosphocreatine, 0.2 U of phosphocreatine kinase]. Enzymatic activity was normalized to protein concentration and control.

2.5 Measurement of intracellular reactive oxygen species formation

After treatment with 0.5 μmol/L MG132 for 48 h, NRCM were loaded with 50 μmol/L dichlorofluorescein diacetate (DCFDA, Invitrogen) at 37°C in the dark for 30 min. DCFDA is cleaved by cellular esterases to non-fluorescent 2′,7′-dichlorofluorescin, which is oxidized by intracellular ROS to the fluorescent product dichlorofluorescein (DCF). Cells were then treated for 30 min with 30 μmol/L H_2O_2 in PBS buffer, trypsinized, and counted. Fluorescence was quantified in a Gemini EM fluorescence plate reader (Molecular Devices, $\lambda_{Ex} = 485$, $\lambda_{Em} = 530$).

2.6 Western blot analysis

Total cell extracts were prepared in RIPA buffer (50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 1% NP40; 0.5% sodiumdeoxycholate; 0.1% SDS) containing Complete™ (Roche Applied Science, Mannheim, Germany) as a protease inhibitor cocktail. Cellular debris was removed by centrifugation. Protein content was determined using the Bradford method (Pierce). Total protein was subjected to SDS–PAGE (10% gels), and polyvinylidene difluoride membranes were probed with antibodies against SOD1 (Abcam, USA), HO1 (Santa Cruz, CA, USA), catalase (Sigma-Aldrich, Germany), or Nrf2 (Santa Cruz). Bands were visualized using the ECL Plus detection system (GE Health Care, Germany). Amido black staining (Sigma-Aldrich) was used as a loading control. Densitometric analyses were performed using ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/).

2.7 Transfections and luciferase assays

NRCM were incubated with DNA/lipofectin (Invitrogen) complexes for 3 h, washed, cultivated in standard medium for 21 h, and then incubated with 0.5 μmol/L MG132 for 48 h. pGL3 luciferase reporter constructs (1.5 μg per well) were driven by wild-type or truncated variants of the human SOD1 promoter.[22] Further analyses were performed employing a construct with a mutated antioxidant response element (ARE) site (TACCATTTCT). Additional experiments included constructs which were solely driven by three ARE sites from SOD1 (TGACATTTCT)[22,23] or one ARE site from HO1 (TGACTCAGC).[24] For co-transfection experiments, we used the luciferase reporter driven by the ARE site of HO1 (1.5 μg per well) and expression vectors of Nrf2 (pCDNA3-mNrf2, 125 ng per well) and kelch-like ECH-associated protein 1 (KEAP1) (pCDNA3-mKEAP1, 125 ng per well when cotransfected with pCDNA3-mNrf2, 250 ng in experiments with MG132). Expression vectors were kindly provided by M. Yamamoto (University of Tsukuba, Japan).[25] To ensure constant amounts of DNA, cells were co-transfected with sufficient amounts of an empty control vector (pCDNA3, Invitrogen) when necessary. Transfection efficiency was controlled by co-transfection of the

renilla luciferase in all experiments (0.5 μg per well). Luciferase assays were performed using the Dual Luciferase Promega Kit (Promega), and data were normalized to the activity of the co-transfected renilla luciferase and control.

2.8 Statistics

Data are expressed as mean $\pm$ SEM, unless otherwise indicated. We calculated significance by t-test or one-way ANOVA where appropriate (SPSS 11.0, Chicago, IL, USA). An error probability of $P < 0.05$ was regarded as significant.

3. Results

3.1 Proteasome inhibition protects cardiac myocytes against H_2O_2-induced cell damage

NRCM were treated with the aldehyde proteasome inhibitor MG132[26] for 48 h followed by the induction of oxidative stress with H_2O_2. The degree of cell damage was analysed by quantifying the release of LDH. The treatment of cardiac myocytes with 0.5 μmol/L MG132 for 48 h did not alter LDH release compared with untreated controls, indicating that low-dose proteasome inhibition is non-toxic (*Figure 1A*). Incubation of untreated myocytes with hydrogen peroxide for 30 min resulted in a six-fold induction of LDH release compared with control cells. In contrast, pre-treatment with proteasome inhibitors markedly reduced H_2O_2-mediated cell damage by >40% within the following 24 h (*Figure 1A*). These data clearly indicate that the treatment of NRCM with non-toxic doses of proteasome inhibitors confers protection against oxidative stress.

Moderate proteasome inhibition was confirmed by the quantification of proteasomal activity: treatment with MG132 for 24 and 48 h reduced the main proteasomal activity—namely the ChT-like activity—by 50 and 35%, respectively (*Figure 1B*).

### 3.2 Proteasome inhibition suppresses intracellular reactive oxygen species formation

To investigate whether this protective effect is accompanied by reduced formation of ROS, NRCM were stained with DCFDA to detect levels of intracellular ROS. Subjecting cardiomyocytes to hydrogen peroxide led to a five-fold induction of oxidant-dependent fluorescence within 30 min (*Figure 2*). However, pre-treatment with MG132 for 48 h reduced this H_2O_2-stimulated ROS increase by half.

3.3 Proteasome inhibition upregulates antioxidative enzymes

In order to elucidate the mechanisms which underlie proteasome inhibitor-mediated protection against oxidative stress, we analysed the expression of antioxidative enzymes in inhibitor-treated cardiac myocytes. The treatment of myocytes with 0.5 μmol/L MG132 induced the expression of several antioxidative enzymes, namely catalase, SOD1, and HO1 in a concerted manner on the protein level (*Figure 3*).

3.4 Proteasome inhibition mediates transcriptional activation of superoxide dismutase 1 via an antioxidant response element

To analyse the underlying mechanisms behind the induction of antioxidative enzymes by proteasome inhibition, we
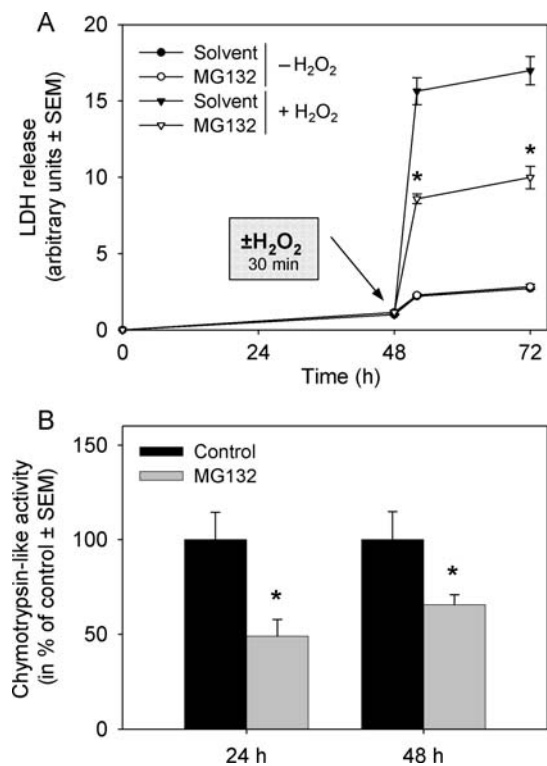
Figure 1 (*A*) Pre-treatment with MG132 protects neonatal rat cardiac myocytes against H_2O_2-mediated cell damage. Myocytes were treated with 0.5 μmol/L MG132 or solvent (DMSO) for 48 h followed by incubation with or without 30 μmol/L H_2O_2 for 30 min. Cell damage was determined by measuring the release of lactate dehydrogenase (LDH) before the addition of H_2O_2, as well as 0, 4, and 24 h after the removal of H_2O_2 ($n = 3$, *$P < 0.05$ vs. control+H_2O_2). (*B*) Treatment of neonatal rat cardiac myocytes with 0.5 μmol/L MG132 for 24 and 48 h results in moderate inhibition of the chymotrypsin-like proteasome activity ($n = 2$, *$P < 0.05$ vs. control).
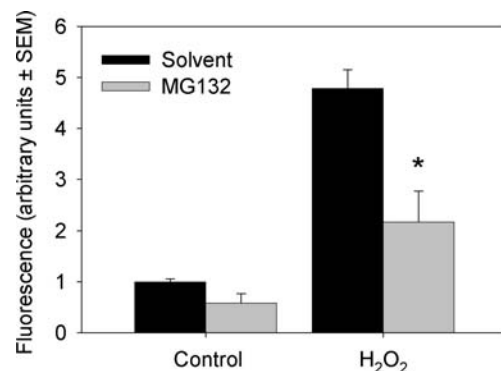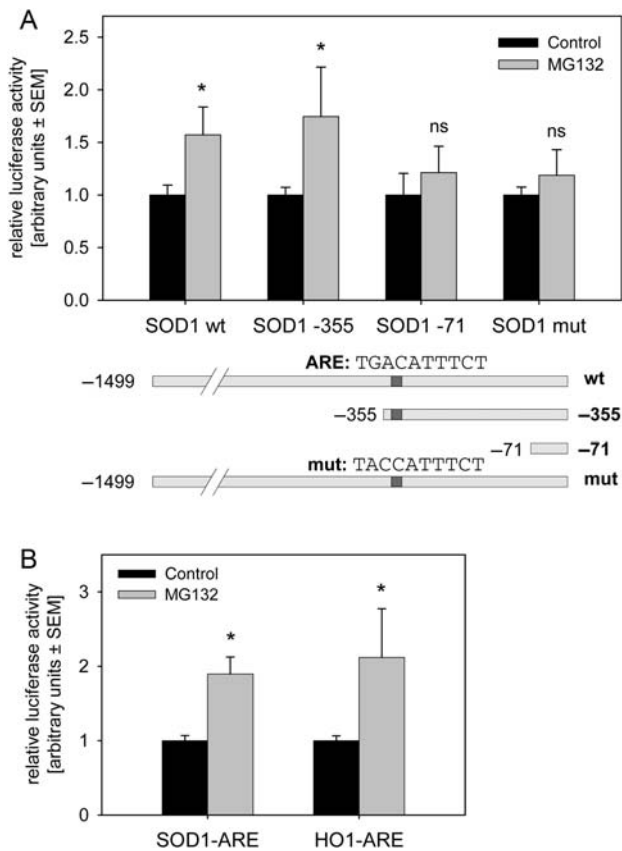
Figure 2 Pre-treatment of neonatal rat cardiac myocytes with 0.5 μmol/L MG132 for 48 h reduces the formation of reactive oxygen species (ROS) after incubation with 30 μmol/L H_2O_2 for 30 min. As a marker for intracellular ROS levels, DCF fluorescence was quantified after the loading of myocytes with 50 μmol/L dichlorofluorescein diacetate (DCFDA) for 30 min ($n = 3$, *$P < 0.05$ vs. solvent control+H_2O_2).

performed reporter gene assays. NRCM were transiently transfected with a luciferase construct driven by the wild-type promoter of SOD1 and then treated with 0.5 μmol/L MG132 or solvent for 48 h. Proteasome inhibition significantly induced luciferase activity, indicating that the induction of antioxidative enzyme expression by MG132 occurs on the transcriptional level (*Figure 4A*).

In order to characterize the proteasome inhibitor-mediated transcriptional effects in further detail, we
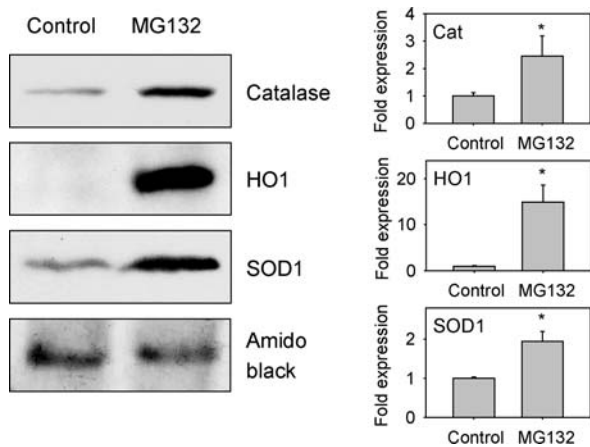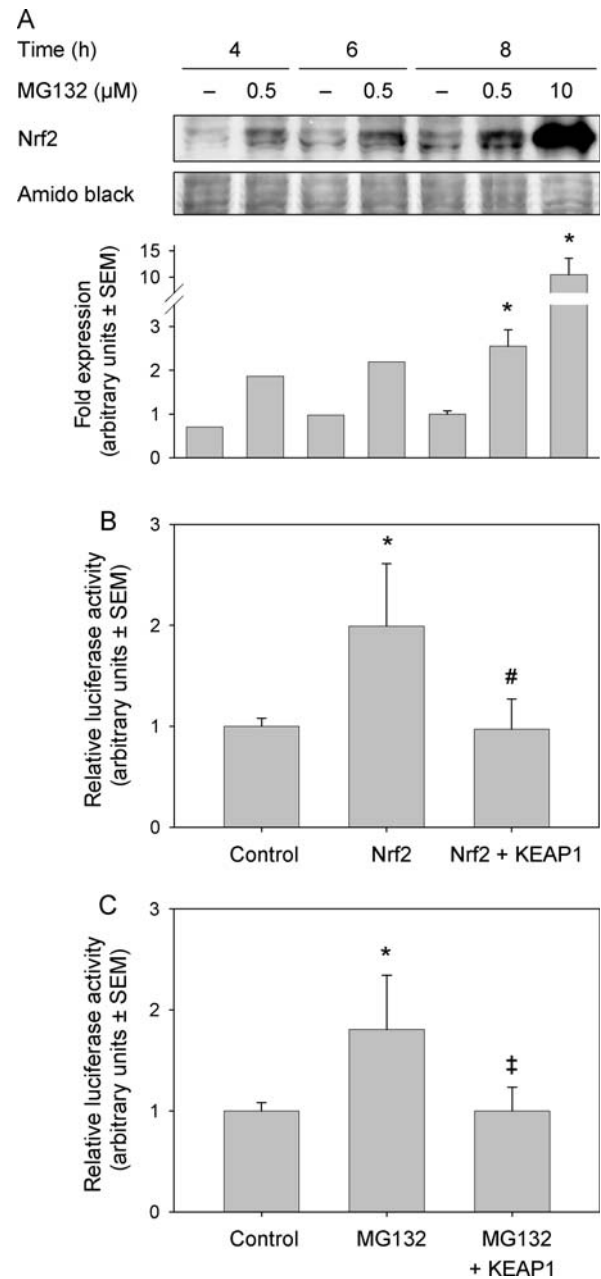
Figure 3 Treatment of neonatal rat cardiac myocytes with 0.5 μmol/L MG132 for 48 h induces protein levels of superoxide dismutase 1 (SOD1), haem oxygenase 1 (HO1), and catalase (Cat). Diagrams show densitometric analyses ($n = 3$, * $P < 0.05$ vs. control).

Figure 4 Treatment of neonatal rat cardiac myocytes with 0.5 μmol/L MG132 results in an antioxidant response element (ARE) site-dependent increase of luciferase activity mediated by the superoxide dismutase 1 (SOD1) promoter. Myocytes were transfected with distinct luciferase constructs driven by the wild-type SOD1 promoter or its mutated variants (A: $n = 4$, * $P < 0.05$ vs. controls) or with vectors solely driven by ARE sites from the promoters of SOD1 or haem oxygenase 1 (HO1) (B: $n = 3$, * $P < 0.05$ vs. control). Luciferase activity was determined 24 h after the addition of MG132.

Figure 5 NF-E2-related factor 2 (Nrf2) protein levels are induced by proteasome inhibition. The inhibition of Nrf2 by the overexpression of kelch-like ECH-associated protein 1 (KEAP1) prevents MG132-mediated induction of antioxidant response element (ARE) site-activity. (A) Treatment of neonatal rat cardiac myocytes with 0.5 μmol/L MG132 for 4 to 8 h increased Nrf2 protein levels. The diagram gives densitometric analyses [$n = 2$–3 (only at 8 h), * $P < 0.05$ vs. control]. (B) Neonatal rat cardiac myocytes were transfected with a luciferase vector driven by an ARE site from the haem oxygenase 1 (HO1) promoter. Cells were co-transfected with an expression vector of Nrf2 and a control vector (pCDNA3) or with equal amounts of expression vectors of Nrf2 and KEAP1. Control cells were co-transfected with an empty control vector (pCDNA3) ($n = 3$, *$P < 0.05$ vs. control, #$P < 0.05$ vs. cells transfected with Nrf2 only). (C) NRCM were co-transfected with the HO1 ARE luciferase reporter and a control vector (pCDNA3) or an expression vector of KEAP1. Cells were then treated with 0.5 μmol/L MG132 for 24 h ($n = 3$, *$P < 0.05$ vs. control, ‡$P < 0.05$ vs. MG132 alone).

performed deletion analyses of the SOD1 promoter. Transfection with a truncated SOD1 promoter construct (residual length of 355 bp) fully retained MG132-mediated induction of luciferase activity. However, further deletion down to −71 bp abolished transcriptional activation by proteasome inhibition, indicating a crucial role for transcription factor-binding sites between −355 and −71 bp (*Figure 4A*). Notably, the responsive segment of the SOD1 promoter contains an ARE, suggesting a possible mode of action for proteasome inhibitor-mediated induction of SOD1 via

transcriptional activation of this element.[23] This notion was confirmed by further transfection experiments employing a luciferase construct which contained a mutated ARE site. Site-directed mutation of the ARE site in the SOD1 promoter completely abolished MG132-mediated upregulation. These data strongly indicate an essential role of the ARE site for transcriptional upregulation of SOD1 by proteasome inhibition (*Figure 4A*).

A highly conserved ARE site is also present in the HO1 promoter. In order to investigate whether ARE sites are sufficient for proteasome inhibitor-mediated induction of antioxidative enzymes, we conducted further experiments employing reporter gene constructs which were solely driven by ARE sites from SOD1 and HO1, respectively. For both constructs, we observed MG132-mediated induction of luciferase activity, suggesting that the identified ARE sites are sufficient for proteasome inhibitor-induced upregulation of antioxidative enzymes (*Figure 4B*).

3.5 Proteasome inhibitor-induced transcriptional activation of antioxidative enzymes depends on the antioxidative transcription factor Nrf2

Genes under the control of ARE sites are known to be activated by the antioxidant transcription factor Nrf2. Nrf2 itself is regulated by the inhibitory protein KEAP1 which mediates ubiquitination and rapid degradation of Nrf2 by the proteasome under normal conditions.[27] Elevated levels of ROS, however, lead to the dissociation of Nrf2 from KEAP1, stabilization of Nrf2, and subsequent transcriptional activation of endogenous antioxidative defence mechanisms.[28] Similarly, the overexpression of Nrf2 induces transcriptional activation, whereas the overexpression of KEAP1 counteracts Nrf2-mediated transcriptional induction.[29]

The treatment of NRCM for 4 to 8 h with 0.5 μmol/L MG132 resulted in a time-dependent increase of Nrf2 protein levels (*Figure 5A*).

In order to elucidate a possible role of Nrf2 in proteasome inhibitor-mediated upregulation of antioxidative enzymes, we co-transfected NRCM with a reporter construct driven by the ARE site from HO1 and expression vectors of Nrf2 and KEAP1. Under control conditions, the co-transfection of myocytes with an Nrf2 expression vector induced luciferase activity, indicating that increased levels of Nrf2 are capable of inducing transcriptional activation via the ARE site of the HO1 promoter. In a proof-of-principle experiment, the co-expression of Nrf2 and KEAP1 blocked Nrf2-induced luciferase activity, demonstrating efficient inhibition of Nrf2-activity by KEAP1 overexpression (*Figure 5B*). In a similar fashion, the overexpression of KEAP1 completely abolished proteasome inhibitor-mediated induction of ARE-dependent luciferase activity (*Figure 5C*). These results suggest that the antioxidative transcription factor Nrf2 is involved in the mediation of transcriptional activation of HO1 upon proteasome inhibition in cardiac myocytes.

3.6 Proteasome inhibitor-mediated induction of antioxidative enzymes and protection against oxidative stress is Nrf2-dependent

To investigate the role of Nrf2 for the protective effects of proteasome inhibition on cardiac myocytes, we performed further experiments using neonatal cardiac myocytes from Nrf2 wild-type and knockout mice. Corroborating our data

from rat cardiac myocytes, the treatment of Nrf2 wild-type myocytes with MG132 for 48 h clearly induced protein levels of HO1, catalase, and SOD1. Importantly, in cardiac myocytes from Nrf2 knockout mice, neither catalase nor HO1 nor SOD1 was upregulated by proteasome inhibition (*Figure 6A*). We further analysed the functional effects of Nrf2-dependent transcriptional activation of antioxidative enzymes on cellular damage of oxidatively stressed myocytes, using LDH assays (*Figure 6B*). Whereas the treatment with MG132 for 48 h protected Nrf2 wild-type cardiac myocytes against H_2O_2-mediated oxidative stress, proteasome inhibition was unable to rescue Nrf2 knockout myocytes as shown by a prominent LDH release of hydrogen peroxide-treated cardiac myocytes (*Figure 6B*). These results suggest a crucial role for Nrf2-mediated transcriptional activation of antioxidative enzymes in proteasome inhibitor-mediated cardioprotection against oxidative stress.

4. Discussion

In this study, we show for the first time that non-toxic proteasome inhibition upregulates antioxidative enzymes in cardiac myocytes via Nrf2-dependent transcriptional activation of ARE sites. The upregulation of several antioxidative enzymes is associated with the reduced formation of ROS and enhanced survival of cardiac myocytes upon the induction of oxidative stress.

As recently published, proteasome inhibition for 48 h by low-dose treatment with MG132 proved to be non-toxic in cardiac myocytes.[30] This was confirmed by a recent report which showed that the inhibition of the proteasome by 2 μmol/L epoxomicin for 24 h—resulting in a reduction of proteasomal activity of ~70%—did not induce apoptosis in cardiac myocytes.[31] Moreover, our present study demonstrates that low-dose proteasome inhibition confers protection of cardiomyocytes against oxidative stress. This is in marked distinction to several previous studies in cardiac myocytes which in general applied high, rather toxic doses in short-term experiments.[19,32] The dose-dependent effects of proteasome inhibition have been analysed by us recently in a systematic manner in endothelial cells. Whereas high doses of proteasome inhibitors induced apoptosis, low-dose proteasome inhibition resulted in a concerted upregulation of several antioxidative enzymes and reduced intracellular ROS formation upon the induction of oxidative stress.[16] In order to reconcile these differential effects of proteasome inhibition, we recently proposed the hypothesis that proteasome inhibitors might serve as poisons or remedies—depending on cell type and applied doses.[20] This model is also supported by data from neuronal cells: partial proteasome inhibition elevated levels of HO1 and glutathione and protected neuronal cells against oxidative stress, whereas high-dose proteasome inhibition induced apoptosis.[17,33–35]

Here, we have extended our previous finding that low-dose proteasome inhibition concertedly upregulates antioxidative enzymes in endothelial cells to cardiomyocytes. The induction of SOD1, HO1, and calatase was observed after 48 h of stimulation with low and non-toxic doses of proteasome inhibitors on the protein (*Figure 3*) and RNA level (data not shown). Although the upregulation of HO1 and SOD1 is very similar in its extent to proteasome inhibitor-mediated induction of these enzymes in
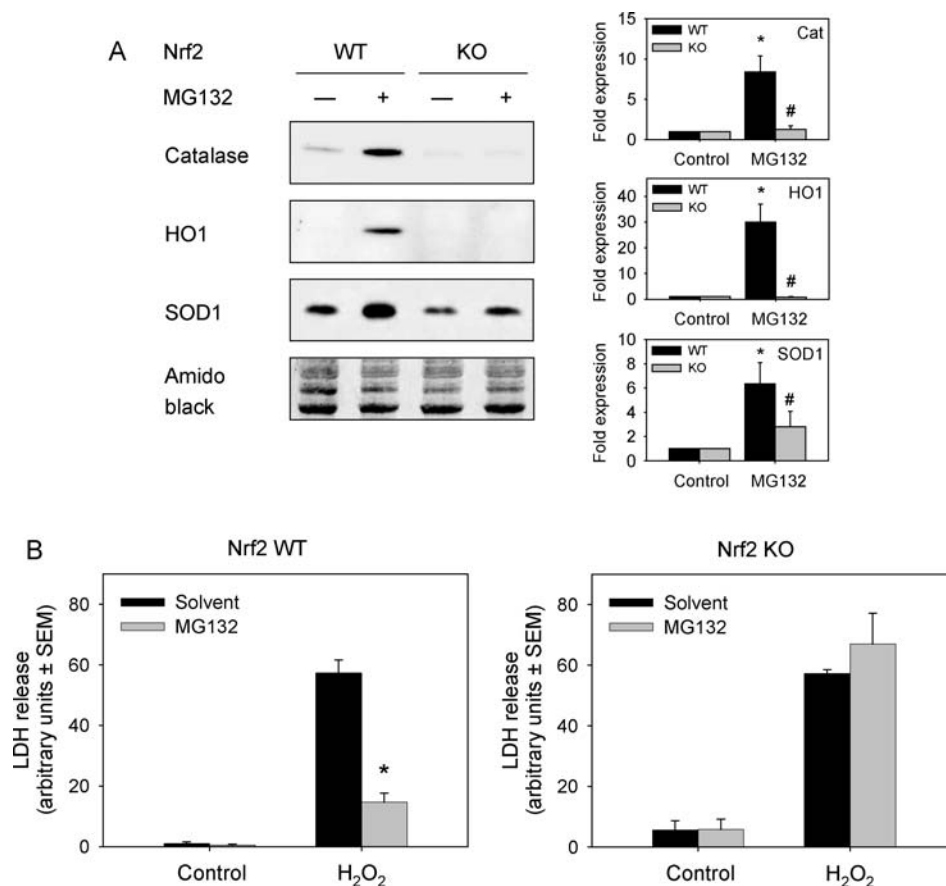
Figure 6 The induction of antioxidative enzymes and protection against oxidative stress of neonatal murine cardiac myocytes by proteasome inhibition are mediated by NF-E2-related factor 2 (Nrf2). (*A*) The expression of superoxide dismutase 1 (SOD1), haem oxygenase 1 (HO1), and catalase (Cat) in cardiac myocytes from Nrf2 wild-type (WT) and knockout (KO) mice after treatment with 0.3 μmol/L MG132 or solvent for 48 h. Diagrams show densitometric analyses ($n = 2$, *$P <$ 0.05 vs. WT control, #$P <$ 0.05 vs. WT MG132). (*B*) Cardiac myocytes were treated with 0.3 μmol/L MG132 or solvent for 48 h followed by incubation with or without 100 μmol/L H_2O_2 for 30 min. Cell damage was determined by measuring the release of lactate dehydrogenase (LDH) 24 h after the removal of H_2O_2 ($n = 3$, *$P <$ 0.05 vs. control).

endothelial cells, we failed to detect significant induction of glutathione peroxidase-3 (GPx-3). Instead of GPx-3, we observed the upregulation of catalase, an enzyme that is—quite similar to GPx-3—involved in the reduction of hydrogen peroxide and organic hydroperoxides.[36] We were able to delineate the mechanisms for the induction of antioxidative enzymes by proteasome inhibition and determined a crucial role for ARE sites in the transcriptional regulation of SOD1 by proteasome inhibition. Comprehensive deletion and mutation of the SOD1 promoter revealed that the ARE site is essential as well as sufficient to mediate the induction of SOD1 by proteasome inhibitors. ARE sites are known to be regulated by the antioxidative transcription factor Nrf2.[18] Under physiological conditions, Nrf2 is bound to its inhibitor KEAP1, which mediates rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome.[27] High ROS levels, however, result in the stabilization of Nrf2 and, consequently, transcriptional activation of numerous endogenous antioxidants via ARE sites.[28] The stabilization of Nrf2 by proteasome inhibition and subsequent transcriptional activation of glutathione S-transferases and glutathione have been shown in different cell types in earlier studies.[17,25,37–39] Here, we observed the induction of Nrf2 protein levels and Nrf2-dependent transcriptional activation of antioxidative enzymes in cardiac myocytes upon treatment with low and non-toxic doses of proteasome inhibitors.

The interplay of Nrf2 and KEAP1 can be mimicked by the transfection of expression vectors. Whereas the overexpression of Nrf2 results in the upregulation of endogenous antioxidants, the overexpression of KEAP1 counteracts Nrf2-dependent gene expression as also shown here for cardiac myocytes (*Figure 5A*).[24,29,40] Interestingly, the overexpression of KEAP1 in cardiac myocytes completely abolished proteasome inhibitor-mediated induction of ARE-mediated luciferase activity. Most probably, the overexpression of KEAP1 leads to rapid (re-)ubiquitination of Nrf2, which has accumulated because of impaired proteasomal degradation, thereby facilitating preferential degradation of Nrf2 by the partially inhibited proteasome. These observations prompted us to conduct further experiments with cardiac myocytes from Nrf2 wild-type and knockout mice: whereas catalase, HO1, and SOD1 were again upregulated by proteasome inhibition in Nrf2 wild-type myocytes, no induction was detected in myocytes from Nrf2 knockout mice. Notably, the failure to induce antioxidative enzymes by treatment with MG132 correlated well with a loss of protection against H_2O_2-mediated oxidative stress in cardiac myocytes from Nrf2 knockout mice. Taken together, our observations clearly indicate an essential role for Nrf2 in the mediation of proteasome inhibitor-dependent up-regulation of antioxidative enzymes and subsequent cardioprotection.

Acute ROS formation plays a critical role in tissue damage upon ischaemia–reperfusion injury.[3,41,42] Consequently, targeting ROS formation has been proposed as a promising therapeutic strategy. However, several approaches which were able to diminish tissue damage in animal or cell culture models are either not applicable to humans or failed to be beneficial in clinical trials.[43–46] Notably, these studies tried to counteract ROS formation by administering 'exogenous' antioxidants such as *N*-acetylcysteine or vitamin E. In contrast, our data give evidence that low-dose proteasome inhibition induces 'endogenous' antioxidative defence mechanisms. Whereas the induction of antioxidative enzymes has also been shown for other substances, e.g. melatonin and D3T,[47,48] proteasome inhibitors exert additional beneficial effects that contribute to cardioprotection. In a porcine model of myocardial infarction, Pye *et al.*[49] reported that the systemic administration of the proteasome inhibitor PS-519 significantly reduced infarction size and preserved regional myocardial function. This effect was mainly attributed to the inhibition of NFκB activation. Similar results were obtained by Campbell *et al.*[50] in an isolated perfused rat heart model of ischaemia–reperfusion. Moreover, we and others have shown previously that proteasome inhibitors induce heat shock proteins in cardiac myocytes.[19,32] In a papillary muscle model of hypoxia, pre-treatment with MG132 for 90 min resulted in enhanced recovery of contractile parameters and increased cardiac myocyte survival.[19,20]

In conclusion, because of their multiple beneficial effects, proteasome inhibitors represent a broad spectrum approach targeting several different pathways that contribute to cardioprotection. Proteasome inhibition might thus represent a promising tool for pharmacological preconditioning of cardiac myocytes.

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