Protection of vascular cells from oxidative stress by proteasome inhibition depends on Nrf2

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Received 30 April 2009; revised 22 July 2009; accepted 10 August 2009; online publish-ahead-of-print 13 August 2009

Time for primary review: 34 days

Aims

Increased levels of reactive oxygen species cause oxidative stress and severely damage lipids, proteins, and DNA. We have previously shown that partial proteasome inhibition induces an antioxidative gene pattern in endothelial cells. Here, we elucidate the mechanisms of proteasome inhibitor-mediated upregulation of antioxidative enzymes and cytoprotection.

Methods and results

Non-toxic proteasome inhibition upregulated mRNA and protein expression of superoxide dismutase 1 (SOD1) and haem oxygenase 1 (HO1) in several human endothelial and vascular smooth muscle cell types. Transcriptional activation of these enzymes was shown by inhibition of RNA polymerase II and nuclear run-on assays. Transfection of endothelial cells with luciferase reporter constructs revealed that upregulation can be largely confined to an antioxidant response element (ARE), which proved to be sufficient for transcriptional activation of SOD1 and HO1. Co-transfection studies and bandshift analyses confirmed binding of the antioxidative transcription factor NF-E2-related factor 2 (Nrf2)—which was stabilized by proteasome inhibition as shown by immunoblots—to the ARE site of HO1. Experiments with aortic endothelial and smooth muscle cells from Nrf2 wild-type and knockout mice revealed an essential role of Nrf2: in wild-type cells, proteasome inhibitor-mediated induction of SOD1 and HO1 was accompanied by protection of vascular cells against oxidative stress as determined by lactate dehydrogenase release assays. In contrast, proteasome inhibitor-mediated induction of antioxidative enzymes and cytoprotection were completely lost in cells from Nrf2 knockout mice.

Conclusion

Nrf2-dependent transcriptional activation of antioxidative enzymes is crucial for proteasome inhibitor-mediated protection of vascular cells against oxidative stress.

Keywords

Oxidative stress • Endothelial cells • Vascular smooth muscle cells • Proteasome inhibitor • Nrf2 • Antioxidant response element • Preconditioning

1. Introduction

Despite ample scientific efforts and major therapeutic advances within the last decades, atherosclerosis and its sequela continue to be the leading cause of death in industrialized countries. As atherogenesis is caused by several independent factors, it is evident that efforts aiming at its prevention or treatment must seek to combat as many contributing factors as possible.1,2 While great progress has been made in the treatment of risk factors such as hypertension and hypercholesteremia, no feasible approaches addressing oxidative stress have reached the clinical routine as yet.3,4 In atherogenesis, highly oxidated lipids contribute to endothelial damage thereby initiating a chronic inflammatory response within the vessel wall. Lipids are oxidatively modified by reactive oxygen species (ROS), e.g. hydrogen peroxide, superoxide anions, and hydroxyl radicals that are metabolic by-products of physiological as well as pathological processes. Cellular ROS are detoxified by a variety of antioxidative enzymes such as superoxide...
dismutase 1 (SOD1), catalase, and haem oxygenase 1 (HO1). Supraphysiological levels of ROS which exceed the capacity of the cellular radical-scavenging systems cause oxidative stress and activate pro-inflammatory pathways. Furthermore, superoxide anions react with vascular nitric oxide to generate high levels of peroxynitrite which in consequence severely diminishes NO bioavailability.3 In addition, peroxynitrite causes nitration of tyrosine which disrupts protein function.6–8 Thereby, ROS contribute to endothelial dysfunction and subsequent formation of atherosclerotic lesions.9 Accordingly, targeting elevated ROS levels represent a promising approach in the prevention of cardiovascular disease. Indeed, in vitro studies as well as small in vivo trials suggest that treatment with antioxidants,10–13 chelators,14 or antioxidative enzymes15 might prevent ROS-mediated damage. However, the most feasible approach—oral supplementation of antioxidants—failed to show beneficial effects in large clinical trials.4,16,17

The ubiquitin proteasome system is the central protein degradation mechanism in eukaryotic cells. It is involved in the regulation of protein quality control,18 intracellular signalling pathways,19–21 and cell cycle.22,23 Accordingly, alteration of its activity represents a promising strategy for the treatment of various human diseases. In 2003, the first proteasome inhibitor, bortezomib (velcade®), was approved by the FDA for the treatment of multiple myeloma. Other inhibitors are currently being under investigation in clinical trials.24 Besides the pro-apoptotic effects of high-dose proteasome inhibition, low-dose proteasome inhibition has been shown to mediate beneficial effects in the absence of toxicity.25 In particular, proteasome inhibitors conferred protection from ischaemia/reperfusion injuries in several different organ and animal models.26,27 These protective effects of proteasome inhibitors have been mainly attributed to suppressed activation of the inflammatory transcription factor NFκB.28 In addition, we and others observed atheroprotective effects in vivo and in vitro: we were able to show that low-dose and non-toxic proteasome inhibition upregulates eNOS but downregulates ET-1 thereby improving endothelial function of aortic rings.29,30 Proteasome inhibition attenuated hypertension in DOCA salt sensitive rats and improved endothelial function in a hypercholesteremic pig model.31,32 We and others have also shown proteasome inhibitor-mediated protection from oxidative stress in endothelial and neuronal cell types.30,33–35 Notably, the protective effects of low-dose proteasome inhibition were associated with uniform upregulation of several antioxidative enzymes. Accordingly, we have recently proposed the hypothesis that proteasome inhibitors may, depending on the dose, serve as both poisons and remedies.25

The aim of our current study was to investigate the underlying mechanisms of proteasome inhibitor-mediated cytoprotection in various vascular cell types.

2. Methods

2.1 Cell culture and experimental conditions
Human umbilical vein endothelial cells (HUVEC) from different donors were isolated and cultured as described previously.37 Cell preparation conformed with local university Ethics guidelines and the principles outlined in the Declaration of Helsinki.36 Human aortic smooth muscle cells (HAoSMC) were purchased from Provitro and kept in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% foetal calf serum (FCS, Biochrom) and 1% penicillin/streptomycin (Invitrogen). Human aortic endothelial cells (HAEC) were obtained from Promocell and cultivated in the presence of a specific endothelial cell growth medium (EC Medium, Promocell) containing 0.4% ECGS/H, 2% FCS, 0.1 mg/mL epidermal growth factor, 1 μg/mL hydrocortisone, 1 ng/mL basic fibroblast growth factor (Promocell), and 1% penicillin/streptomycin. Murine aortic endothelial cells (MAEC) were prepared from adult mice as described previously37 and cultivated in a 1:1 mixture of MCD131 and DMEM (Invitrogen) containing 5% FCS (Biochrom). Vascular smooth muscles cells (mVSMC) were prepared from adult mice as described previously38 and cultivated in DMEM containing 10% FCS and 1% penicillin/streptomycin. Cell preparation conformed with local university guidelines and 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).

All cells were cultivated at 37°C in a humidified atmosphere at 5% CO2. For proteasome inhibition, cells were incubated with 70 nmol/L (HUVEC, MAEC) or 200 nmol/L (HAEC, HAoSMC, mVSMC) MG132 (Biomol) or solvent (0.1% DMSO, Merck) for up to 24 h. Cell type-specific doses were used to ensure partial proteasome inhibition (~50% in chymotrypsin-like activity assays) which was non-toxic and did not activate caspase-3 activity—a hallmark of apoptosis (data not shown).

2.2 Real-time RT–PCR analysis
Preparation of total RNA and reverse transcription was performed as recently described.29 The PCR primers for amplification of human and mouse HO1 and SOD1 and the housekeeping genes human ribosomal protein L19 and murine hypoxanthin-phosphoribosyl-transferase cDNAs were obtained from TIB MOLBIOL. Quantitative amplification of the HO1 and SOD1 cDNA was performed using the SYBR Green method as described previously.29 mRNA expression was standardized to the housekeeping gene whose expression remained unaffected by our experimental conditions. Expression of HO1 and SOD1 after proteasome inhibition was normalized to expression under control conditions (0.1% DMSO) by means of the comparative Ct method (2-ΔΔCt). α-Amanitin (1 mg/mL stock solution in water) was obtained from Roche Applied Science.

2.3 Western blot analysis
Trypsinized cells were lysed 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) as protease inhibitor. After centrifugation to remove cellular debris, protein concentration was quantified using the Bradford method (Pierce). Equal amounts of proteins were subjected to SDS–polyacrylamide gel electrophoresis and membranes were probed with antibodies against NF-E2-related factor 2 (Nrf2) (sc-722x, Santa Cruz), HO1 (sc-1797, Santa Cruz), SOD1 (ab16831, Abcam), and β-actin (Cell Signalling). Bands were visualized using the ECL plus detection system (GE Health Care). For Nrf2 immunoblots, protein samples were prepared from nuclear extracts (Nuclear Extract Kit, ActiveMotif) and amido black staining (Sigma-Aldrich) served as a loading control (data not shown).

2.4 Nuclear run-on assay
Nuclear run-on assays were performed as previously described by Zhang et al.39 with minor modifications. Briefly, HUVEC were

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treated with 70 nmol/L MG132 for 12 h and intact nuclei were prepared. Cells were lysed in the presence of 5% NP40 and then resuspended (40% glycerol; 50 mmol/L Tris–HCl, pH 7.4; 5 mmol/L MgCl2; 0.1 mmol/L EDTA, pH 8). For run-on assays, nuclei were resuspended in x 1 reaction buffer (5 mmol/L Tris–HCl, pH 8; 2.5 mmol/L MgCl2; 150 mmol/L KCl) containing 2.5 mmol/L ATP, GTP, CTP (Roche), and 2 mmol/L Biotin-UTP (Ambion). Nuclear transcription was allowed to take place for 45 min at 30°C. After digestion of nuclear DNA, nuclear RNA was prepared by addition of TRIZOL (Invitrogen) and biotinylated RNA was enriched using streptavidin beads (ActiveMotif). After pelleting of the beads, the supernatant was retained as it contains non-biotinylated nuclear RNA which served as the input control. Beads were washed once in 2 × SSC containing 15% formamide, once in 2 × SSC and then resuspended in aqua dest. for subsequent RT–PCR. Input RNA, newly synthesized HO1 and SOD1 mRNA were quantified by real-time RT–PCR.

2.5 Transfections and luciferase assays
HUVEC were transiently transfected using lipofectamine (Invitrogen) according to the manufacturer’s protocol. pG3 luciferase reporter constructs were driven by wild-type or truncated variants of the human SOD1 promoter or by the antioxidant response element (ARE) sites from SOD1 or HO1. The sequences of all primers, constructs, and probes used are given in Supplementary material online, Table S1. For cotransfection of an HO1-ARE reporter and a KEAP1 expression vector, sufficient amounts of an empty control vector (pCDNA3, Invitrogen) were added to the transfection reaction to ensure constant amounts of DNA. To control transfection efficiency, the pcDNA4.74 renilla luciferase plasmid (Promega) was cotransfected with DMSO (0.1%) or 70 nM MG132 for 24 h. Luciferase activity was measured in transfected cells for 20 h. Cells were then stimulated either with DMSO (0.1%) or 70 nM MG132 for 12 h and intact nuclei were resuspended in 100 μL 2xSSC containing 15% formamide, once in 2xSSC and then resuspended in aqua dest. for subsequent RT–PCR. Input RNA, newly synthesized HO1 and SOD1 mRNA were quantified by real-time RT–PCR.

2.6 Nuclear extracts and bandshift analysis
Nuclear extracts of HUVEC were prepared with the Nuclear Extract Kit by ActiveMotif. DNA oligonucleotides containing one copy of the HO1-ARE site (see Supplementary material online, Table S1) were obtained from TIB MOBIOL and labelled with [γ-32P]-ATP (Hartmann Analytic). One micro litre labelled oligonucleotides and 10 μg nuclear proteins were incubated in reaction buffer [1 μg poly(dI-dC) (Roche Molecular Biochemicals), 5 mmol/L DT, 15 mmol/L HEPES, pH 7.9; 1 mmol/L EDTA; 10% glycerol, 2 μg BSA Fraction V (Sigma)] for 30 min at 30°C. Samples were subjected to a 5% polyacrylamide gel using 0.25 x Tris-borate-EDTA buffer at 200 V. For supershift analysis, samples were incubated with 2 μg of the Nrf2 antibody (sc-722x, Santa Cruz) before separation by electrophoresis for 2 h at 4°C after the binding reaction. Labelled oligonucleotides were detected by autoradiography.

2.7 Quantification of cell damage
MAEC and mVSMC from Nrf2 wild-type and knockout mice were pretreated with 70 or 200 nmol/L MG132, respectively, for 24 h. Oxidative stress was then induced by incubation with 500 μmol/L H2O2 for 1 h. Cellular damage was quantified 4 h (mVSMC) or 24 h (MAEC) after removal of H2O2 by measuring the release of lactate dehydrogenase (LDH). LDH concentrations were determined using the CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega).

2.8 Statistics
Data are expressed as mean ± standard error of mean. Significance was calculated by t-test or one-way ANOVA where appropriate (SPSS 11.0, Chicago, IL, USA). We regarded an error probability of P < 0.05 as significant.

3. Results

3.1 Proteasome inhibition upregulates expression of HO1 and SOD1 in human vascular cells
HUVEC as well as HAoSMC and endothelial cells (HAEC) from human aortas were treated with non-toxic doses of the proteasome inhibitor MG132. Western blots and RT–PCR analyses revealed increased protein and mRNA levels of both HO1 and SOD1 in all investigated cell types 24 h after proteasome inhibition (Figure 1A and B). Cotreatment of HUVEC with the transcriptional inhibitor α-amanitin (2.5 μg/mL) completely abolished proteasome inhibitor-induced upregulation of SOD1 and HO1 indicating de novo mRNA synthesis upon proteasome inhibition (Figure 1C). This was confirmed by nuclear run-on experiments: stimulation of HUVEC with 70 nmol/L MG132 for 12 h induced HO1 and SOD1 mRNA by four- and three-fold, respectively, when compared with the input RNA control. A similar extent of upregulation was observed when we monitored synthesis of newly transcribed mRNA by RT–PCR analysis of biotinylated RNA (Figure 1D). These data clearly indicate that proteasome inhibition upregulates both HO1 and SOD1 by transcriptional activation.

3.2 Proteasome inhibition mediates transcriptional activation of SOD1 and HO1 via an ARE
Proteasome inhibition in HUVEC transfected with a luciferase reporter construct driven by the promoter of SOD1 significantly induced luciferase activity by more than 2-fold. Transfecting HUVEC with truncated variants of the SOD1 promoter identified a crucial promoter segment between −355 and −71: whereas deletion of the first 1144 bp preserved inducibility of luciferase activity by MG132, further deletion down to −71 bp led to a complete loss of proteasome inhibitor-mediated transcriptional activation (Figure 2A). Notably, the essential promoter segment contains an ARE. A highly conserved ARE site is also found in the HO1 promoter. Remarkably, transfection of constructs driven solely by the ARE sites of SOD1 and HO1, respectively, revealed that both ARE sites are sufficient to mediate induction of luciferase activity by MG132 (Figure 2B).
3.3 Proteasome inhibition mediates stabilization and binding of Nrf2 to the HO1-ARE site

ARE sites are activated by the antioxidant transcription factor Nrf2. Using immunoblot analysis, we observed nuclear accumulation of Nrf2 after only 3 h of proteasome inhibition. Stabilization of Nrf2 peaked at ~6 h and returned to near baseline levels within 24 h. Notably, sustained proteasome inhibition using 10 μmol/L MG132 induced nuclear Nrf2 levels to a markedly higher extent compared with low-dose proteasome inhibition (Figure 3A).

In bandshift analyses, we then investigated the pattern of proteins bound to the HO1-ARE site upon proteasome inhibition. Binding of complex I to the HO1-ARE site was enhanced only 30 min after low-dose proteasome inhibition and further increased within 24 h (Figure 3B). The same was observed for the much weaker binding of complex II to the HO1-ARE site. Binding of complex I and complex II was also enhanced after 6 h of sustained proteasome inhibition with 10 μmol/L MG132 (Figure 3B). Specificity of DNA binding was confirmed by competition analysis (data not shown).

Next, we performed supershift analyses to identify Nrf2 as part of the complexes bound to the HO1-ARE site under conditions of proteasome inhibition. Addition of an anti-Nrf2 antibody to the binding reaction altered the migration pattern of the HO1-ARE site only under conditions of proteasome inhibition: in the presence of 70 nmol/L MG132, we observed a weak but detectable supershift that was absent in the DMSO-treated control. The supershifted complex was more prominent in the presence of high inhibitor doses of 10 μmol/L MG132 (Figure 3C). These data clearly indicate that Nrf2 is recruited to the conserved HO1-ARE site under conditions of proteasome inhibition.

Figure 1  Low-dose proteasome inhibition induces expression of SOD1 and HO1 in human vascular cells. (A) Immunoblots of HO1 and SOD1 after treatment with solvent, 70 nmol/L MG132 (HUVEC) or 200 nmol/L MG132 (HAEC, HAoSMC) for 24 h. (B) Treatment with MG132 for 24 h stimulated mRNA expression of SOD1 and HO1 in HUVEC, HAEC, and HAoSMC (*P < 0.05 vs. solvent control, n = 9–12). (C) Cotreatment with 2.5 μg/mL α-amanitin abolished MG132-mediated induction of SOD1 and HO1 in HUVEC (*P < 0.05 vs. HUVEC without α-amanitin cotreatment, n = 3–6). (D) Nuclear run-on experiments indicate induction of de novo synthesis of HO1 and SOD1 mRNA in HUVEC treated with 70 nmol/L MG132 for 12 h (*P < 0.05 vs. control, n = 3).
Nrf2 is regulated in its activity by an inhibitory protein called kelch-like ECH-associated protein 1 (KEAP1) which is responsible for ubiquitination and subsequent proteasomal degradation of Nrf2. Overexpression of KEAP1 leads to rapid degradation of Nrf2. In order to investigate the role of Nrf2 in transcriptional activation of HO1 by proteasome inhibition, we tested the effect of KEAP1 overexpression on activation of the HO1-ARE site reporter. Notably, overexpression of KEAP1 significantly attenuated MG132-induced activation of the HO1-ARE site (Figure 3D), suggesting involvement of Nrf2 in transcriptional activation of HO1 by proteasome inhibition.

3.4 Induction of SOD1 and HO1 by proteasome inhibition depends on Nrf2

In order to prove the essential role of Nrf2 for proteasome inhibitor-mediated activation of HO1 and SOD1, we prepared smooth muscle and endothelial cells from Nrf2 wild-type and knockout mice. Similar to human endothelial cells, mouse aortic endothelial cells (MAEC) upregulated endogenous HO1 and SOD1 mRNA level by 8- and 2-fold, respectively, in response to partial proteasome inhibition. In contrast, transcriptional activation of both HO1 and SOD1 was completely abrogated in MAEC from
Nrf2 knockout mice (Figure 4A). Similarly, proteasome inhibitor-induced RNA expression of HO1 and SOD1 in mVSMC was lost in mVSMC from Nrf2-deficient mice (Figure 4B). These results were further corroborated by immunoblot analysis of HO1 and SOD1 (Figure 4C): proteasome inhibitor-induced upregulation of HO1 and SOD1 was completely abrogated in endothelial as well as in smooth muscle cells from Nrf2 knockout mice.

### 3.5 Proteasome inhibitor-mediated protection against oxidative stress depends on Nrf2

The above data strongly suggest a causal role for Nrf2 in upregulation of HO1 and SOD1 by proteasome inhibition. We therefore investigated whether stabilization of Nrf2 is a prerequisite for proteasome inhibitor-mediated protection of vascular cells against oxidative stress. mVSMC and MAEC from Nrf2 wild-type and knockout mice were stressed with 500 μmol/L hydrogen peroxide for 1 h and cellular damage was monitored by measuring the release of LDH after 4 and 24 h, respectively. In the presence of Nrf2, hydrogen peroxide induced pronounced damage of MAEC as shown by a more than 40-fold increase in LDH release (Figure 5A). In the absence of Nrf2, however, these protective effects of the proteasome inhibitor MG132 were lost (Figure 5A). Similarly, protection from hydrogen peroxide-induced cellular damage was...
achieved in mVSMC by non-toxic proteasome inhibition, but was absent in cells from Nrf2 knockout mice (Figure 5B). These data clearly indicate an essential role of Nrf2 for proteasome inhibitor-mediated cytoprotection.

4. Discussion

Recently, we have shown that low-dose and non-toxic proteasome inhibition induces an antioxidative gene pattern in HUVEC. Here, we give evidence that this effect is not limited to endothelial cells but in fact is detectable in a variety of vascular cell types. Importantly, induction of antioxidative enzymes is associated with protection against hydrogen peroxide-mediated oxidative cell damage in both endothelial cells and mVSMC. Furthermore, we were able to identify Nrf2-dependent activation of AREs as the causal link between proteasome inhibition and cytoprotection.

Our present study gives insights into the underlying mechanisms of how non-toxic proteasome inhibition induces an antioxidative defence response in vascular cells. Inhibition of RNA polymerase II-dependent induction of SOD1 and HO1 by α-amanitin, activation of reporter gene constructs, and nuclear run-on experiments clearly revealed that this response involves transcriptional upregulation of antioxidative enzymes by proteasome inhibition (Figures 1 and 2). Deletion analyses of the SOD1 promoter identified a crucial segment, which contains an ARE. The ARE sites from both SOD1 and HO1 proved to be sufficient for proteasome inhibitor-mediated transcriptional activation. In bandshift and supershift analyses, we confirmed binding of the antioxidative transcription factor Nrf2 to the highly conserved HO1-ARE site upon proteasome inhibition (Figure 3).

Nrf2 is a central player in the regulation of cellular defence mechanisms against environmental stresses. In the absence of oxidative stress, the adaptor protein KEAP1 links Nrf2 to the ubiquitination machinery and mediates rapid degradation of Nrf2 by the proteasome. High levels of ROS, however, induce dissociation of KEAP1 from Nrf2 thereby liberating and stabilizing Nrf2. Nrf2 is then able to enter the nucleus and orchestrate the antioxidant defence response of the cell by the activation of numerous protective genes. Here, we demonstrate the essential role for Nrf2 in the transcriptional activation of antioxidative enzymes by proteasome inhibition. First, we observed diminished transcriptional activation of the ARE site from HO1 by overexpression of KEAP1 (Figure 3D). Secondly, we obtained genetic evidence using endothelial and smooth muscle cells from Nrf2 wild-type and knockout mice: transcriptional activation of both SOD1 and HO1 was completely abolished in Nrf2-deficient vascular cells. Importantly, loss of Nrf2 not only abrogated transcriptional activation of antioxidative enzymes but also severely diminished the cytoprotective effects of low-dose proteasome inhibition.
Proteasome inhibitor-mediated and Nrf2-dependent expression of endogenous antioxidants such as glutathione S-transferases and glutathione have previously been demonstrated in several different cell types.34,43–50 Yamamoto et al.34 reported induction of γ-glutamylcysteine synthetase and subsequent protection of neurons by proteasome inhibition. MG132-mediated protection of astrocytes from oxidative injury was mediated by increased expression of HO1.33 In two recent studies, we showed a protective antioxidative response upon proteasome inhibition in cardiac myocytes and in isolated rat aortas. In the latter study, non-toxic proteasome inhibition prevented TNFα-induced vascular dysfunction by reduction of superoxide production.46,47 Levonen et al.53 demonstrated that overexpression of Nrf2 activated expression of antioxidant enzymes in rat mVSMC and significantly reduced oxidative stress in rat aortas in vivo.

Notably, the protective role of Nrf2 is not restricted to the antioxidative stress response: the use of Nrf2 knockout cells in comparative microarray analyses has revealed a vast number of Nrf2 oxidative stress response: the use of Nrf2 knockout cells in comparative microarray analyses has revealed a vast number of Nrf2.

Moreover, activation of Nrf2 is crucial for the protective effects of laminar shear stress on endothelial cells.54,55 Induction of antioxidative enzymes (e.g. HO1 and ferritin) and reduced expression of the pro-inflammatory molecules monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1 was shown to be dependent on Nrf2.56,57 Taken together, these data strongly support the notion that Nrf2 acts as a central regulator of cellular stress and survival.58

In distinction to clinical studies—which tried to counteract oxidative stress by supplementation of exogenous antioxidants—induction of endogenous antioxidants via activation of the Nrf2/ARE pathway might represent a physiological and thus more promising antioxidative strategy. Notably, the induction of SOD1 by Nrf2 is not prone to nitrosylation which is associated with a decrease in enzyme activity.

As the pathogenesis of atherosclerosis involves multiple steps such as endothelial dysfunction, enhanced oxidative stress, and increased inflammation, the anti-inflammatory effects of proteasome inhibitors and their ability to improve endothelial dysfunction might further contribute to the anti-atherogenic potential of proteasome inhibition.26,29 To test this hypothesis, we are currently evaluating the effects of proteasome inhibition in an animal model of atherosclerosis.

Supplementary material
Supplementary Material is available at Cardiovascular Research online.

Acknowledgements
We are grateful to Andrea Weller and Kerstin Daemen for their excellent technical assistance.

Conflict of interest: none declared.

Funding
Part of this work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to K.S. and S.M. [DFG Nrf2/STA567/4-1]. S.M. was a research fellow of the Charité—Universitätsmedizin Berlin. K.W. has been supported by the DFG Graduiertenkolleg 865.

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