

## RESEARCH PAPER

# Tolerance to nitroglycerin through proteasomal down-regulation of aldehyde dehydrogenase-2 in a genetic mouse model of ascorbate deficiency

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## BACKGROUND AND PURPOSE

L-gulonolactone oxidase-deficient (*Gulo*<sup>-/-</sup>) mice were used to study the effects of ascorbate deficiency on aortic relaxation by nitroglycerin (GTN) with focus on changes in the expression and activity of vascular aldehyde dehydrogenase-2 (ALDH2), which catalyses GTN bioactivation.

## EXPERIMENTAL APPROACH

Ascorbate deficiency was induced in *Gulo*<sup>-/-</sup> mice by ascorbate deprivation for 4 weeks. Some of the animals were concomitantly treated with the proteasome inhibitor bortezomib and effects compared with ascorbate-supplemented *Gulo*<sup>-/-</sup>, untreated or nitrate-tolerant wild-type mice. Aortic relaxation of the experimental groups to GTN, ACh and a NO donor was studied. Changes in mRNA and protein expression of vascular ALDH2 were quantified by qPCR and immunoblotting, respectively, and aortic GTN denitration rates determined.

## KEY RESULTS

Like GTN treatment, ascorbate deprivation induced vascular tolerance to GTN that was associated with markedly decreased rates of GTN denitration. Ascorbate deficiency did not affect ALDH2 mRNA levels, but reduced ALDH2 protein expression and the total amount of ubiquitinated proteins to about 40% of wild-type controls. These effects were largely prevented by ascorbate supplementation or treating *Gulo*<sup>-/-</sup> mice with the 26S proteasome inhibitor bortezomib.

## CONCLUSIONS AND IMPLICATIONS

Our data indicate that ascorbate deficiency results in vascular tolerance to GTN via proteasomal degradation of ALDH2. The results support the view that impaired ALDH2-catalysed metabolism of GTN contributes significantly to the development of vascular nitrate tolerance and reveal a hitherto unrecognized protective effect of ascorbate in the vasculature.

## Abbreviations

ALDH2, aldehyde dehydrogenase-2; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; GDN, glycerol dinitrate; GTN, glycerol trinitrate (nitroglycerin); *Gulo*, L-gulonolactone oxidase; WT, wild type; XO, xanthine oxidase

## Introduction

Organic nitrates, in particular nitroglycerin (GTN), have been used for decades to treat angina pectoris, but development of tolerance limits their usefulness and precludes continuous administration to patients. The proposed mechanisms underlying the development of vascular tolerance to GTN are manifold, including impaired bioactivation to NO or a related activator of soluble guanylate cyclase (Sage *et al.*, 2000; Chen *et al.*, 2002), desensitization of soluble guanylate cyclase (Schröder *et al.*, 1988; Sayed *et al.*, 2008) and oxidative stress associated with increased generation of superoxide in blood vessels leading to limited NO bioavailability due to peroxynitrite formation (Münzel *et al.*, 1995). Although challenged by some studies (Hinz and Schröder, 1998; Csont *et al.*, 2002; Hanspal *et al.*, 2002), the oxidative stress concept is supported by several reports on beneficial effects of antioxidants like ascorbate in experimental and clinical studies of nitrate tolerance (Bassenge *et al.*, 1998; Watanabe *et al.*, 1998; McVeigh *et al.*, 2002). This hypothesis is questioned, however, by a study showing that ascorbate did not prevent tolerance development (Milone *et al.*, 1999).

There is a large body of evidence indicating that aldehyde dehydrogenase-2 (ALDH2) is a key enzyme of vascular GTN bioactivation and that exposure of blood vessels to GTN causes mechanism-based inactivation of ALDH2, resulting in impaired GTN bioactivation (Mayer and Beretta, 2008). Despite general agreement on the essential role of ALDH2 inactivation in nitrate tolerance, this concept has been questioned in a recent study showing that the haemodynamic response recovered more rapidly after GTN treatment than total vascular ALDH activity (D'Souza *et al.*, 2011). Although GTN bioactivation is catalysed by cytosolic rather than mitochondrial ALDH2 (Beretta *et al.*, 2012), exposure of blood vessels to the nitrate appears to result in mitochondrial oxidative stress. The oxidative stress concept has been combined with impaired GTN biotransformation in a unifying hypothesis explaining nitrate tolerance and endothelial dysfunction upon exposure of blood vessels to GTN (Münzel *et al.*, 2011).

We have recently shown that ascorbate deprivation of guinea pigs causes vascular hyposensitivity to GTN (Wölkart *et al.*, 2008; Wenzl *et al.*, 2009). Despite the apparent involvement of impaired ALDH2-catalysed GTN biotransformation in both classical nitrate tolerance and GTN hyposensitivity induced by ascorbate deprivation, there was a remarkable difference between the two animal models. While residual GTN-triggered relaxation of nitrate-tolerant aortas was not further aggravated by ALDH2 inhibitors, the GTN response of ascorbate-deficient blood vessels was still sensitive to ALDH2 inhibition (Wölkart *et al.*, 2008; Wenzl *et al.*, 2009). Lack of antibodies and sequence information precluded further studies on the molecular mechanisms underlying this striking difference of the two nitrate tolerance models in guinea pigs.

Unlike guinea pigs and humans, which do not express L-gulonolactone oxidase (Gulo), the terminal enzyme of ascorbate biosynthesis (Linster and Van Schaftingen, 2007), mice and rats synthesize the vitamin endogenously, making them independent of dietary ascorbate. This is a major problem for researchers aiming to study the consequences of ascorbate deficiency in those rodents. To overcome this

problem, we created mice that are unable to synthesize ascorbate by targeted deletion of the Gulo-encoding gene (Maeda *et al.*, 2000) and found that impaired vasorelaxation to GTN in ascorbate deficiency is associated with down-regulation of vascular ALDH2 expression. In search for the underlying mechanism, we observed that ALDH2 mRNA levels were not significantly altered and, therefore, studied proteasomal degradation of ALDH2 as potential culprit. Taking into account that ascorbate deficiency may cause oxidative stress in the vasculature, we tested for reduced response of aortic rings to ACh and NO as a functional marker for superoxide generation, measured mRNA expression of xanthine oxidase (XO) and NADPH oxidases, and determined the redox status of the mice.

## Methods

### *Mice and experimental groups*

Female and male Gulo<sup>(-/-)</sup> mice (Maeda *et al.*, 2000) on C57BL/6 background were housed at the local animal facility in approved cages and kept on a regular 12 h dark/light cycle. They were fed standard mouse chow diet (Altromin 1324, containing <40 mg·kg<sup>-1</sup> ascorbate; Königshofer Futtermittel Ebergassing, Austria) and provided drinking water *ad libitum*. Drinking water was supplemented with 330 mg ascorbate per litre and 0.01 mM EDTA, and renewed twice per week. This treatment was shown to provide enough ascorbate to prevent scurvy and maintain weight and health in Gulo<sup>(-/-)</sup> mice (Maeda *et al.*, 2000; Parsons *et al.*, 2006). At 3–4 months of age, Gulo<sup>(-/-)</sup> mice were randomly divided into three groups. While one group was left on ascorbate supplementation (ascorbate-supplemented), ascorbate deficiency was induced in the other groups by feeding ascorbate-free diet for 4 weeks (ascorbate-deficient). At the time point of ascorbate deprivation, one group of Gulo<sup>(-/-)</sup> mice was intraperitoneally injected the proteasome inhibitor bortezomib (0.5 mg·kg<sup>-1</sup> body weight; Selleck Chemicals, Houston, TX, USA) once every 3 days over the 4 weeks deprivation period (bortezomib-treated ascorbate-deficient; Van Herck *et al.*, 2010). For comparison, classical nitrate tolerance was induced in age-matched wild-type (WT; C57BL/6) mice by subcutaneous injection of GTN (20 mg·kg<sup>-1</sup>·per day; b.i.d.) for 4 days as described (Griesberger *et al.*, 2011). WT mice kept on standard mouse chow and non-supplemented drinking water served as controls. At the end of the treatment period, animals were anaesthetized with 5% isoflurane and thereafter killed by cervical dislocation. Depth of anaesthesia was monitored by toe pinch reflex test. The descending aorta was removed and used for functional studies and determination of GTN denitration, latter measured as formation of 1,2- and 1,3- glycerol dinitrate (GDN). For determination of ascorbate and protein expression levels, tissues were frozen in liquid nitrogen and stored at -70°C until analysis. Animals were handled in accordance with the Austrian law on experimentation with laboratory animals (last amendment, 2004), which is based on the US National Institutes of Health guidelines. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

### Relaxation of aortic rings

For determination of isometric tension of aortic rings, a previously described protocol (Wölkart *et al.*, 2008) was slightly modified. Rings of ~3 mm width were mounted in 5 mL organ baths filled with oxygenated Krebs–Henseleit solution (composition in mM: NaCl 118.4, NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, D-glucose 10.1; pH 7.4). Care was taken to retain the endothelium for relaxation experiments. Tissues were equilibrated for 60 min by repeatedly adjusting tension to 0.5 g and changing the bath solution. Bathing solution was then replaced by a depolarizing solution containing 100 mM K<sup>+</sup> for determination of maximal contraction. Rings that did not elicit adequate and stable contraction to high K<sup>+</sup> were considered as damaged and discarded. After washout, tissues were precontracted with the thromboxane mimetic 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F<sub>2</sub> $\alpha$  (U-46619; Sigma; Vienna, Austria) to an equivalent level of ~1.2 g (i.e. ~90% of maximal contractile activity). Notably, vasoconstrictor response to 50 nM U-46619 did not significantly differ between experimental groups [mean total tone of all rings per group: 1.16  $\pm$  0.02 g (WT), 1.14  $\pm$  0.03 g (ascorbate-supplemented) and 1.17  $\pm$  0.02 g (ascorbate-deficient,  $n = 14$  mice per group)]. After reaching stable contraction, cumulative concentration-response curves were established using ACh (1 nM–10  $\mu$ M; Sigma), GTN [1 nM–100  $\mu$ M; Nitropol® ampoules (4.4 mM); G. Pohl-Boskamp GmbH, Hohenlockstedt, Germany] or 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO; 1 nM–10  $\mu$ M; Alexis Corp., Lausen, Switzerland). Some rings were incubated for 45 min with chloral hydrate (5 mM) before addition of agonists to test for the involvement of ALDH2 in GTN bioactivation. Contractile force corresponding to each concentration of the agonists was recorded and expressed as percent of precontraction (=baseline).

### Determination of ascorbate levels

Plasma as well as homogenates of aortas and liver were acidified with meta-phosphoric acid and ascorbate levels measured by HPLC and UV detection at 264 nm using standard methods (Karlsen *et al.*, 2007; Wenzl *et al.*, 2009).

### GTN denitration and ALDH2 protein expression in aorta

Vascular denitration of GTN to 1,2- and 1,3-GDN was determined by incubation of aortic slices with <sup>14</sup>C-GTN and quantification of metabolites by radio thin-layer chromatography and liquid scintillation counting as described previously for guinea pig aorta (Wenzl *et al.*, 2009). For determination of ALDH2 protein expression, sliced mouse aortas were homogenized with a glass potter in 50 mM Tris-HCl, 0.5 mM EDTA, 0.25 M sucrose buffer (pH 7.2), containing 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche Diagnostics GmbH, Graz, Austria). After centrifugation at 200 $\times$   $g$  at 4°C for 5 min, protein concentration was determined with a BCA protein assay kit (Thermo Scientific, purchased via THP, Vienna, Austria). For immunoblotting, 25  $\mu$ g of total protein was separated on 12% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. For detection of ALDH2 or  $\beta$ -actin, membranes were blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20

(vol/vol), while the membranes used for detection of ubiquitinated proteins were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (vol/vol). After blocking, blots were incubated overnight with a primary polyclonal antibody to human ALDH2 (1:20 000 – kindly provided by Dr Henry Weiner), to  $\beta$ -actin (1:200 000 – Sigma-Aldrich, Vienna, Austria) or to ubiquitin (1:4000 – Dako Österreich GmbH, Vienna, Austria), washed and incubated for 1 h with a HRP-conjugated anti-rabbit (ALDH2 or ubiquitin) or anti-mouse ( $\beta$ -actin) IgG secondary antibody (both 1:5000 – Sigma-Aldrich). Bands were visualized with ECL Prime Western blot detection reagent (GE Healthcare, purchased via VWR, Vienna, Austria) using an E.A.S.Y. 1.3 HC camera (Herolab GmbH, Wiesloch, Germany) to detect ALDH2 and  $\beta$ -actin. For detection of ubiquitinated proteins, blots were exposed to X-ray films and all bands above 54 kDa used for quantification. Data are expressed as % of WT.

### RNA extraction, reverse transcription and gene expression analysis

Total RNA was isolated from homogenized aortas using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) including DNase treatment of samples. RNA was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vienna, Austria). qPCR analyses were performed with ~25 ng cDNA using TaqMan® Universal PCR Master Mix and pre-designed TaqMan Gen Expression Assays. Reactions were carried out on a 7300 Real Time PCR System (Applied Biosystems, Vienna, Austria). Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and for 1 min at 60°C. Data were analysed according to the 2<sup>- $\Delta\Delta$ Ct</sup> method using cyclophilin D as reference gene. Lack of amplification was verified in no-template controls.

### Determination of antioxidant status

Plasma total antioxidative status was determined using a commercially available kit (TAS NX2331, Randox, Crumlin, UK) following the manufacturer's instructions. The reaction is based on inhibition of metmyoglobin- and H<sub>2</sub>O<sub>2</sub>-induced oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) by plasma antioxidants (Miller *et al.*, 1993). In addition, the antioxidative capacity of acetonitrile-deproteinized plasma samples was measured using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method described recently (Chrzczanowicz *et al.*, 2008).

### Statistical analysis

Data are presented as mean values  $\pm$  SEM of  $n$  experiments. Concentration-response curves established with different ring segments from a single animal were averaged and counted as individual experiment. Individual concentration-response curves were fitted to a Hill-type model giving estimates of agonist potency (EC<sub>50</sub>) and efficacy (E<sub>max</sub>). To account for the biphasic characteristic of GTN-induced vasorelaxation, EC<sub>50</sub> values were calculated for the high-affinity component (1 nM–1  $\mu$ M GTN). Analysis of variance (ANOVA) with *post hoc* Bonferroni–Dunn test was used for comparison between groups using StatView® (Version 5.0; SAS Institute Inc., Cary, NC, USA). Significance was assumed at  $P < 0.05$ .

## Results

### Body weights and ascorbate levels

In line with previous reports on the *Gulo*<sup>(-/-)</sup> mouse strain (Maeda *et al.*, 2000; Parsons *et al.*, 2006), ascorbate deprivation for 4 weeks caused slight weight loss in *Gulo*<sup>(-/-)</sup> mice as compared to ascorbate-supplemented or WT animals (Table 1). As further shown in the table, ascorbate levels in plasma, aorta and liver were markedly decreased in ascorbate-deficient *Gulo*<sup>(-/-)</sup> mice as compared to WT animals. In plasma and aortas of ascorbate-supplemented *Gulo*<sup>(-/-)</sup> mice, ascorbate levels were similar to WT controls, but in liver, the levels were significantly lower than in WT.

### Effect of ascorbate deficiency on vascular relaxation to GTN

As shown in Figure 1A, GTN caused concentration-dependent relaxation of U-46619-precontracted aortas isolated from WT mice. In line with a previous observation in mice (Chen *et al.*, 2005), the GTN response was biphasic with high- (<1  $\mu$ M; Figure 1B) and low- (>1  $\mu$ M) affinity components. The EC<sub>50</sub> value for the high-affinity component of relaxation of WT aorta was  $40 \pm 6$  nM (unfilled squares in Figure 1B). As expected, the high-affinity component of GTN relaxation was considerably diminished in nitrate-tolerant animals (filled squares in Figure 1B). Similarly, ascorbate deficiency led to fivefold lower relaxing potency of GTN with a corresponding EC<sub>50</sub> value of  $200 \pm 50$  nM (unfilled diamonds in Figure 1B;  $P < 0.05$ ) and a significant decrease in maximal relaxation induced by 1  $\mu$ M GTN ( $E_{\max} = -24 \pm 3\%$  as compared to  $-48 \pm 3\%$  in untreated WT). The low-affinity response to GTN was also reduced in nitrate-tolerant WT and ascorbate-deficient *Gulo*<sup>(-/-)</sup> mice, though the effect was less pronounced. The apparent GTN tolerance of ascorbate-deficient aortas was fully prevented by ascorbate supplementation of *Gulo*<sup>(-/-)</sup> mice (unfilled circles in Figure 1A and B) or proteasome inhibition by bortezomib (filled diamonds in Figure 1A and B). The corresponding EC<sub>50</sub> values for the high-affinity response ( $57 \pm 15$  nM and  $64 \pm 16$  nM, respectively) and maximal relaxation to 1  $\mu$ M GTN ( $E_{\max} = -49 \pm 4\%$  and  $-44 \pm 8\%$ , respectively) were not significantly different from

WT controls. Preincubation with the ALDH inhibitor chloral hydrate caused a pronounced right-ward shift of the GTN concentration-response curve of untreated WT, ascorbate-supplemented or bortezomib-treated ascorbate-deficient *Gulo*<sup>(-/-)</sup> aortas, while the effect of chloral hydrate on relaxation of nitrate-tolerant or ascorbate-deficient blood vessels was either abolished (nitrate-tolerant) or very small in magnitude (ascorbate-deficient; Supporting Information Figure S1).

### Effects of ascorbate deficiency on vascular relaxation to ACh and DEA/NO

Since long-term ascorbate deficiency may cause oxidative stress in the vasculature and compromise NO-mediated vascular relaxation through superoxide generation, we compared relaxation to ACh and exogenously applied NO of untreated or nitrate-tolerant WT aortas with that of blood vessels isolated from ascorbate-deficient, bortezomib-treated ascorbate-deficient and ascorbate-supplemented *Gulo*<sup>(-/-)</sup> mice. As shown in Figure 1C, ACh relaxed untreated WT (open squares), nitrate-tolerant WT (filled squares), ascorbate-deficient (open diamonds), ascorbate-deficient bortezomib-treated (filled diamonds) and ascorbate-supplemented (open circles) aortas with EC<sub>50</sub> values of  $200 \pm 68$ ,  $200 \pm 53$ ,  $260 \pm 68$ ,  $140 \pm 30$  and  $150 \pm 40$  nM, respectively. The corresponding  $E_{\max}$  values were  $-66 \pm 4$ ,  $-59 \pm 4$ ,  $-60 \pm 3$ ,  $-70 \pm 4$  and  $-68 \pm 5\%$ . The differences between experimental groups were not statistically significant, indicating that even 4 weeks of ascorbate deprivation does not cause significant endothelial dysfunction in mice. Figure 1D shows that the response to the direct NO donor DEA/NO was neither affected by ascorbate deficiency or classical nitrate tolerance. The calculated EC<sub>50</sub> values were  $128 \pm 30$ ,  $186 \pm 31$ ,  $199 \pm 88$ ,  $176 \pm 16$  and  $87 \pm 25$  nM for untreated WT, nitrate-tolerant, ascorbate-deficient, bortezomib-treated ascorbate-deficient and -supplemented aortas, respectively.

### Vascular GTN reductase activity

To test for the effect of ascorbate deprivation on vascular GTN biotransformation, we measured the rates of 1,2- and 1,3-GDN formation. Aortic rings of WT and ascorbate-

**Table 1**

Body weight and ascorbate levels in plasma, aorta and liver of ascorbate-deficient and -supplemented *Gulo*<sup>(-/-)</sup> mice in comparison to WT

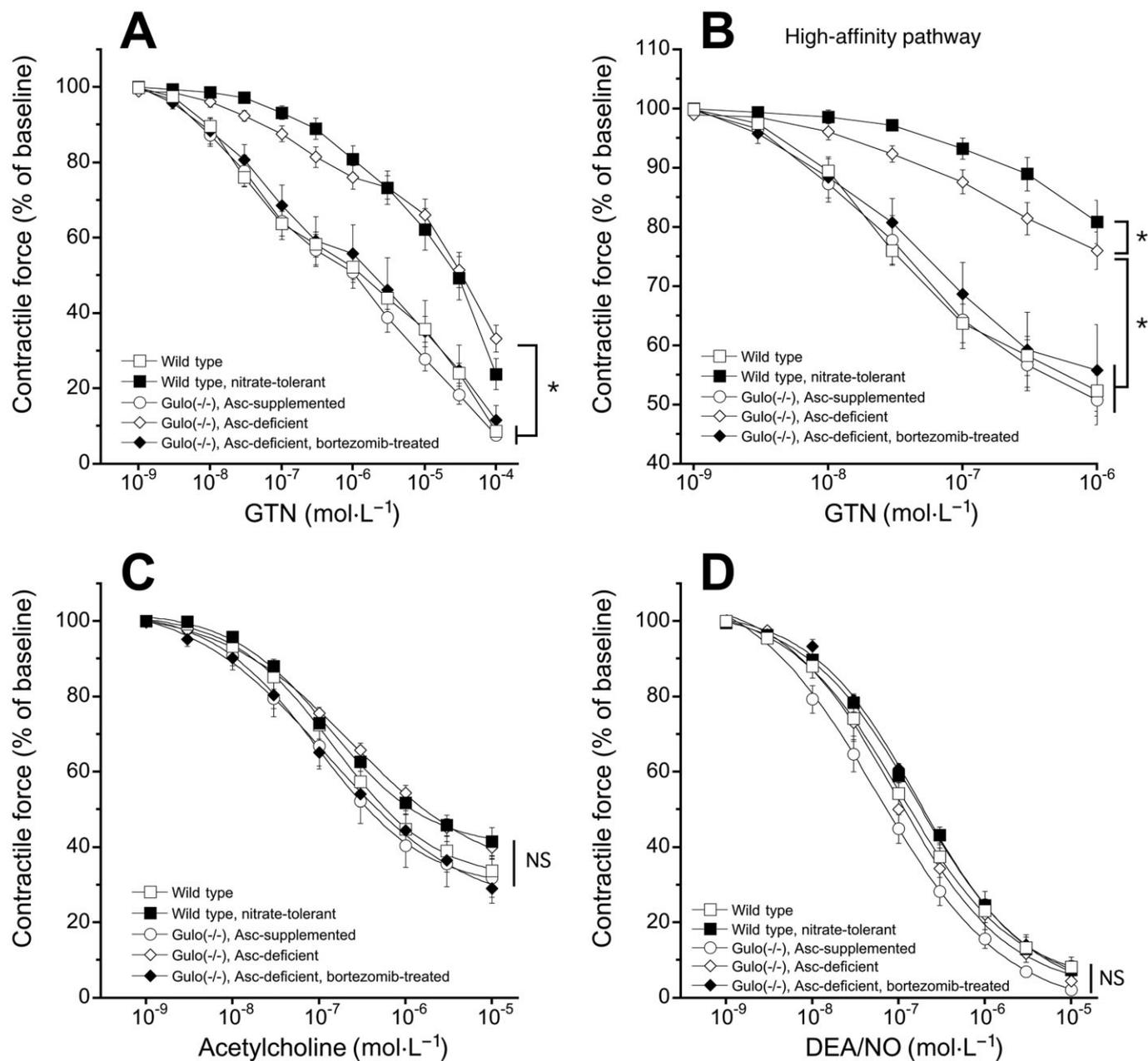
Genotype	WT		<i>Gulo</i> <sup>(-/-)</sup>	
			asc-deficient	asc-supplemented
Body weight (g)	$26 \pm 0.4$		$23 \pm 0.7^1$	$26 \pm 0.7^2$
Plasma ascorbate ( $\mu$ M)	$132 \pm 8.1$		$11 \pm 4.8^1$	$95 \pm 22^2$
Aortic ascorbate (pmol·mg <sup>-1</sup> )	$93 \pm 27$		$5 \pm 2^1$	$74 \pm 14^2$
Liver ascorbate (pmol·mg <sup>-1</sup> )	$710 \pm 50$		$127 \pm 7^1$	$350 \pm 70^{1,2}$

Data are mean values  $\pm$  SEM of 12–15 (body weight), 8 (plasma), 5 (aorta) or 9 (liver) duplicate determinations.

<sup>1</sup> $P < 0.05$  versus WT.

<sup>2</sup> $P < 0.05$  versus ascorbate-deficient (ANOVA).

asc, ascorbate.



**Figure 1**

GTN (A, B), ACh (C) and DEA/NO (D) concentration-response curves of aortic rings from untreated WT (open squares), nitrate-tolerant WT (filled squares), ascorbate-supplemented (open circles), ascorbate-deficient (open diamonds) and bortezomib-treated ascorbate-deficient (filled diamonds) *Gulo*<sup>-/-</sup> mice. For clarity, the high-affinity pathway of the GTN curve is illustrated separately in panel B. Rings were precontracted with the thromboxane mimetic U-46619. In panels A, B and D, data are mean values  $\pm$  SEM of nine (untreated WT, ascorbate-supplemented, ascorbate-deficient) or five (WT, nitrate-tolerant, bortezomib-treated ascorbate-deficient) animals. In panel C, data are mean values  $\pm$  SEM of five animals. Concentration-response curves of different ring segments from a single animal were averaged and counted as individual experiment. \* $P < 0.05$  versus ascorbate-deficient animals. The effect of nitrate tolerance is not indicated for reasons of clarity. NS, non-significant.

supplemented *Gulo*<sup>-/-</sup> mice exhibited similar rates of GTN denitration ( $\sim 0.7$  pmol 1,2-GDN  $\times$  min<sup>-1</sup>  $\times$  g-wet weight<sup>-1</sup>; see Table 2). Formation of the 1,3-isomer was very low, with 1,2-/1,3-GDN ratios of 17.1 and 23.5 for WT and ascorbate-supplemented *Gulo*<sup>-/-</sup> mice, respectively, confirming previ-

ous reports (Chen *et al.*, 2002; Wölkart *et al.*, 2008; Wenzl *et al.*, 2009). Ascorbate deprivation of the mice led to reduction of denitration rates to about 25% of WT controls that was accompanied by a decrease in the 1,2-/1,3-GDN ratio from 22 to 3.4.

**Table 2**

Rates of GTN denitration of aortic rings from WT, ascorbate-supplemented (asc-suppl.) and ascorbate-deficient (asc-def.) *Gulo*<sup>-/-</sup> mice

Genotype	1,2-GDN	1,3-GDN
	(pmol·min <sup>-1</sup> ·g <sup>-1</sup> )	
WT	0.77 ± 0.177	0.05 ± 0.025
<i>Gulo</i> <sup>-/-</sup> , asc-suppl.	0.66 ± 0.162	0.03 ± 0.044
<i>Gulo</i> <sup>-/-</sup> , asc-def.	0.17 ± 0.057 <sup>1,2</sup>	0.05 ± 0.026

Data are mean values ± SEM of four animals.

<sup>1</sup>*P* < 0.05 compared with WT animals.

<sup>2</sup>*P* < 0.05 compared with ascorbate-supplemented mice (ANOVA).

asc, ascorbate.

### Vascular ALDH2 expression

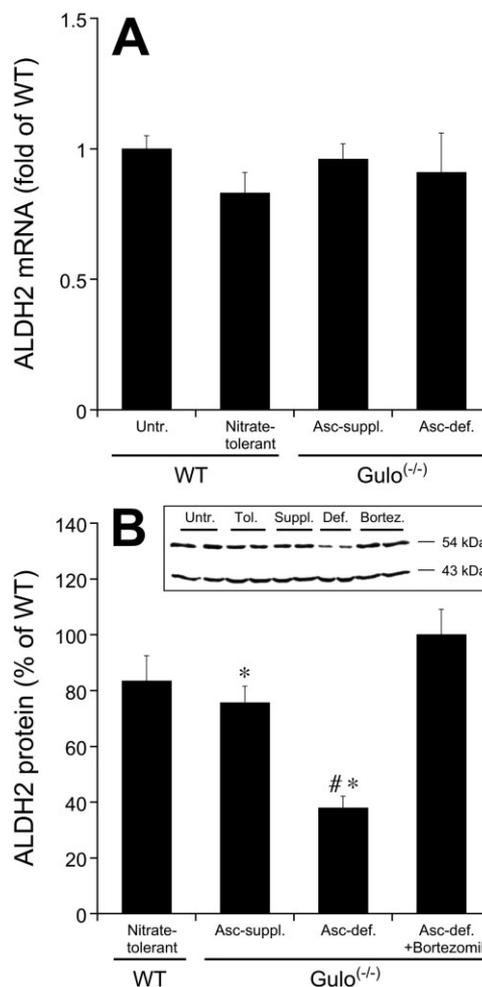
The role of ALDH2 in the development of vascular tolerance to GTN was further studied by measuring ALDH2 mRNA and protein levels in aortas of the different experimental groups. As shown in Figure 2A, ALDH2 mRNA expression was not altered by any experimental intervention, suggesting that neither classical nitrate tolerance nor ascorbate deficiency significantly affected vascular ALDH2 gene transcription. However, ascorbate deprivation of *Gulo*<sup>-/-</sup> mice for 4 weeks reduced aortic ALDH2 protein levels to 38% of WT controls (Figure 2B). This effect was largely reversed by ascorbate supplementation or treatment with bortezomib. In contrast, classical nitrate tolerance led to a much less-pronounced decrease in aortic ALDH2 protein expression (83.4% of WT). This value agrees well with most published data including results obtained with human arterial and venous blood vessels (Hink *et al.*, 2007; Szöcs *et al.*, 2007; Wenzel *et al.*, 2007). However, one study found that ALDH2 expression was markedly reduced to 20% of controls in vena cava of nitrate-tolerant rats (D'Souza *et al.*, 2011).

### Effect of ascorbate deficiency on vascular levels of ubiquitinated proteins

Since the protective effect of bortezomib suggested that the loss of ALDH2 is due to activation of the proteasome in ascorbate-deficient blood vessels, we measured the levels of ubiquitinated proteins in aortic lysates. As shown in Figure 3, the total amount of ubiquitinated proteins was reduced in ascorbate-deficient aortas to 37 ± 8.0% of untreated WT controls and this effect was fully restored by ascorbate supplementation or treating *Gulo*<sup>-/-</sup> mice with bortezomib. Note that the reduction in ubiquitinated protein levels in ascorbate-deprived aortas was virtually identical to the degree of ALDH2 down-regulation (cf. Figures 2B and 3). However, it should be noted that signal intensity of the inevitably over-exposed lanes may have been underestimated due to lack of linear response.

### Vascular mRNA levels of NADPH oxidases (NOX2 and NOX4) and XO

Considering the function of ascorbate as major endogenous antioxidant, we measured mRNA expression of potential

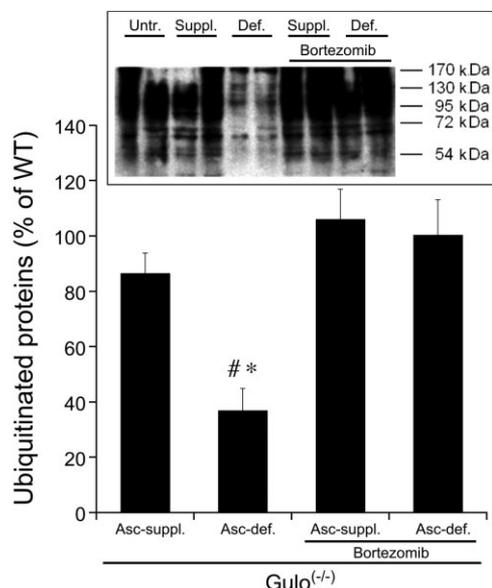
**Figure 2**

Aortic ALDH2 mRNA (A) and protein (B) levels of nitrate-tolerant WT, ascorbate-supplemented (Asc-suppl.), ascorbate-deficient (Asc-def.) and bortezomib-treated ascorbate-deficient *Gulo*<sup>-/-</sup> aortas relative to untreated WT controls. mRNA levels were analysed according to the 2<sup>-ΔΔCt</sup> method using cyclophilin D as reference gene. ALDH2 protein expression was analysed by immunoblotting and quantified densitometrically with band intensities of samples from untreated WT mice (applied on the same gels) set to 100%. A representative blot is shown as inset. Data are mean values ± SEM of four (A) or 6–9 (B) animals. \**P* < 0.05 compared with untreated (non-tolerant) WT animals. #*P* < 0.05 compared with ascorbate-supplemented mice.

sources of vascular superoxide production, that is, NADPH oxidases (NOX2 and NOX4) and XO in aortas of ascorbate-deficient and -supplemented *Gulo*<sup>-/-</sup> mice in comparison to WT. There was no change in the expression levels of XO, NOX2 or NOX4 mRNAs in any experimental group (see Supporting Information Table S1).

### Redox state

As shown in Figure 4, two established methods provided no evidence for a significant change in the systemic redox status of ascorbate-deficient *Gulo*<sup>-/-</sup> mice as compared to WT or ascorbate-supplemented controls.



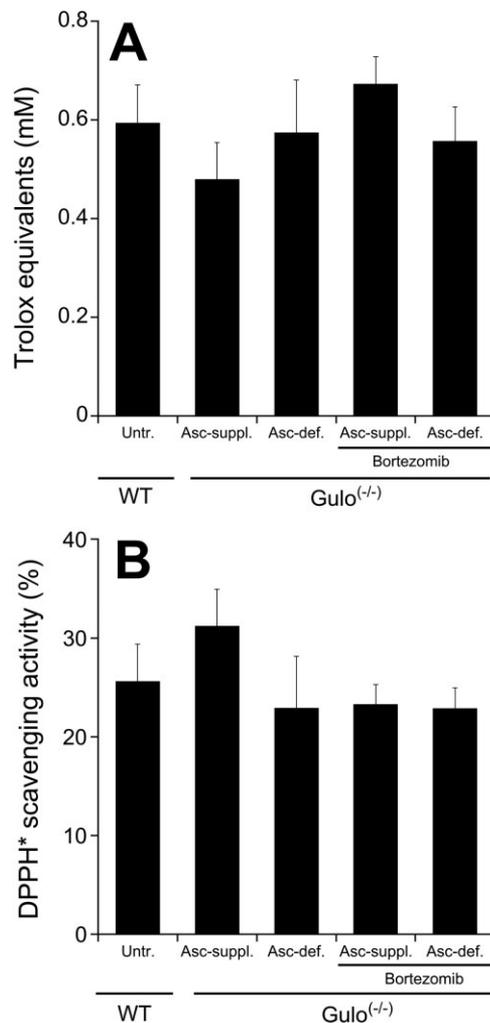
**Figure 3**

Levels of ubiquitinated proteins in lysates of aortas from ascorbate-supplemented (Asc-suppl.) and ascorbate-deficient (Asc-def.) *Gulo*<sup>-/-</sup> mice with and without bortezomib treatment relative to untreated WT controls. A representative blot is shown as inset. All bands >54 kDa (MW of ALDH2) were included in the densitometric quantification. Average band intensities of samples from untreated WT mice (applied on the same gels) were set to 100%. Data are mean values  $\pm$  SEM of five animals. \* $P < 0.05$  compared with untreated WT animals. # $P < 0.05$  compared with ascorbate-supplemented mice.

## Discussion and conclusions

In the present study, we used the *Gulo*<sup>-/-</sup> genetic mouse model to clarify the mechanism underlying the development of nitrate tolerance in ascorbate deficiency described previously in guinea pigs (Wölkart *et al.*, 2008; Wenzl *et al.*, 2009). Upon 4 weeks of feeding with ascorbate-free diet, ascorbate plasma levels of *Gulo*<sup>-/-</sup> mice were reduced to approximately 20% of ascorbate-supplemented controls or WT (see Table 1). Decreased ascorbate plasma levels were associated with impaired relaxation of isolated aortas in response to GTN (see Figure 1).

The organ bath experiments showed that the high-affinity response to GTN was abolished in both classical nitrate tolerance and upon ascorbate deprivation of *Gulo*<sup>-/-</sup> mice, whereas relaxation of blood vessels from ascorbate-supplemented mice was identical to WT (see Figure 1B). The responsiveness of aortic rings to ACh and DEA/NO was not affected by ascorbate deprivation (see Figure 1C and D). Together with the about fourfold reduced rates of 1,2-GDN formation measured in ascorbate-deficient aortas (see Table 2), these results indicate that ascorbate deprivation interferes specifically with GTN bioactivation. Thus, the hyposensitivity of ascorbate-deficient blood vessels to GTN resembles classical nitrate tolerance, which is also associated with reduced vascular ALDH2 activity (Sydow *et al.*, 2004; Wölkart *et al.*, 2008). However, while exposure of blood vessels to GTN results in mechanism-based inactivation of



**Figure 4**

Redox status of ascorbate-supplemented (Asc-suppl.) and ascorbate-deficient (Asc-def.) *Gulo*<sup>-/-</sup> mice with and without bortezomib treatment relative to untreated WT controls. (A) Total antioxidant status of plasma was determined using a commercially available kit as described in Methods. Results are expressed as Trolox equivalents. (B) Antioxidant capacity of plasma samples was measured as 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity. 100% refers to complete loss of DPPH absorbance at 550 nm, that is, complete reduction of the radical. Data are mean values  $\pm$  SEM of plasma samples obtained from five mice.

ALDH2 (Mayer and Beretta, 2008) with only 20–30% reduction of protein levels [see Figure 2B and (Hink *et al.*, 2007; Szöcs *et al.*, 2007; Wenzl *et al.*, 2007)], ascorbate deficiency led to markedly reduced vascular ALDH2 protein levels. Surprisingly, this effect was not accompanied by reduced ALDH2 mRNA expression (see Figure 2A), pointing to a post-transcriptional mechanism. Significant reduction of ubiquitinated protein levels (see Figure 3) suggested that activation of the proteasome might cause degradation of ALDH2 in ascorbate-deprived aortas. This hypothesis was tested with bortezomib, a selective inhibitor of the 26S proteasome (Papandreou, 2005). Indeed, treatment with bortezomib prevented all effects of ascorbate deprivation, that is, the

decrease in ubiquitinated protein levels, ALDH2 down-regulation and the development of nitrate tolerance, strongly suggesting that impaired GTN bioactivation in ascorbate-deficient aortas was due to proteasomal degradation of vascular ALDH2. This is apparently not a consequence of ALDH2 inactivation because the effect of classical nitrate tolerance, which leads to inactivation of the enzyme, was much less pronounced. Moreover, inhibition of the proteasome with bortezomib completely prevented the effects of ascorbate deficiency and led to normal relaxation response to GTN, indicating that ALDH2 was active in the ascorbate-deficient vessels.

It is well established that the ubiquitin-proteasome system is critically involved in the regulation of cardiovascular function and various pathologies (Willis *et al.*, 2010). An important function of the proteasome appears to be degradation of oxidized proteins formed in conditions of oxidative stress (Shang and Taylor, 2011). Effects of ascorbate on proteasomal activity have not been reported so far, but ascorbate supplementation was shown to prevent protein ubiquitination caused by oxidative stress in atherogenesis (Hermann *et al.*, 2003). Since ubiquitination targets proteins towards proteasomal degradation, this may reflect a related mechanism by which vascular ascorbate protects blood vessels from oxidative injury. Considering the function of ascorbate as endogenous antioxidant and the role of oxidative stress in activation of the proteasome, we carefully studied whether ascorbate deprivation of *Gulo*<sup>(-/-)</sup> mice led to systemic or vascular oxidative stress. A previous study found that the plasma antioxidant capacity of ascorbate-deficient *Gulo*<sup>(-/-)</sup> mice was significantly reduced as compared to ascorbate-supplemented controls (Maeda *et al.*, 2000). However, using spectrophotometric methods instead of the chemiluminescence technique used by Maeda *et al.*, we observed no changes of the antioxidant status or the antioxidative capacity of the plasma (see Figure 4). It has been reported that the assays are sensitive to different factors and yield divergent results upon ascorbate infusion (Waring *et al.*, 2003), rendering it difficult to judge which measurements correctly reflect the redox status of the animals. The results of the present study suggest that an antioxidative defence system efficiently compensates the systemic lack of ascorbate *in vivo*. These data do not exclude local oxidative stress in the vasculature, but normal relaxation to DEA/NO and ACh as well as unchanged NADPH oxidase and XO expression levels appear to exclude significant formation of superoxide in ascorbate-deficient blood vessels. Of note, these measurements were made in the absence of GTN, which is known to trigger oxidative stress in the vasculature and the oxidative stress response could be enhanced under conditions of ascorbate deficiency. Thus, despite the lack of experimental evidence, we cannot exclude a role of oxidative stress with certainty. In addition, it is conceivable that activation of the proteasome involves selective oxidation of highly redox-sensitive proteins that are normally protected by ascorbate. It should be noted that we cannot exclude that bortezomib acted through increased expression of antioxidant enzymes as has been reported for a rat model of alcoholic liver disease (Bardag-Gorce *et al.*, 2011).

Taken together, our results reveal a novel mechanism of vascular tolerance to GTN that is caused by ascorbate deprivation.

In contrast to classical nitrate tolerance, which essentially involves oxidative inactivation of ALDH2 by GTN, ascorbate deficiency leads to impaired GTN bioactivation through proteasomal degradation of ALDH2. Since nitrate tolerance was only observed upon severe ascorbate deprivation, it appears unlikely that the therapeutic effects of GTN are compromised in ascorbate-deficient subjects. However, regulation of ALDH2 expression could contribute to the well-documented beneficial effects of ascorbate in nitrate tolerance (Mayer and Beretta, 2008) and disorders associated with overproduction of reactive aldehydes that are detoxified by ALDH2 (Chen *et al.*, 2008). Future work is necessary to address these issues and to clarify the molecular mechanism underlying activation of the proteasome in ascorbate-deprived blood vessels.

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## Conflict of interest

None declared.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1** Effect of chloral hydrate (CH, 5 mM, filled symbols) on the GTN concentration-response of aortic rings from untreated WT (squares), nitrate-tolerant WT (triangles), ascorbate-supplemented (circles), ascorbate-deficient (diamonds) and bortezomib-treated ascorbate-deficient (right-angled triangles) *Gulo(-/-)* mice (A). For clarity the high affinity pathway of the GTN response in the absence or

presence of CH for each experimental group is illustrated separately in panels B-F (up to 1  $\mu$ M GTN). Aortic rings were incubated for 45 min with CH before the addition of GTN. Data are mean values  $\pm$  SEM of 9 (untreated WT, ascorbate-supplemented, ascorbate-deficient) or 5 (WT, nitrate-tolerant, bortezomib-treated ascorbate-deficient) animals. Concentration-response curves of different ring segments from a single animal were averaged and counted as individual experiment. \* $P < 0.05$ . NS, non-significant

**Table S1** Aortic mRNA expression of NOX2, NOX4, and XO in wildtype (WT), ascorbate-supplemented (asc-suppl.) and ascorbate-deficient (asc-def.) *Gulo(-/-)* mice.