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Heterozygous Deficiency of Manganese Superoxide Dismutase in Mice (Mn-SOD$^{+/-}$) as a Novel Approach to Assess the Role of Oxidative Stress for the Development of Nitrate Tolerance

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Running title: Effect of Mn-SOD deficiency on nitrate tolerance

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Abbreviations: GTN, glyceryl trinitrate (nitroglycerin); ACh, acetylcholine; ALDH-2, mitochondrial aldehyde dehydrogenase; Cu,Zn-SOD, copper/zinc superoxide dismutase (cytosolic and extracellular isoforms); DHE, dihydroethidine; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; Mn-SOD, manganese superoxide dismutase (mitochondrial isoform); Mn-SOD$^{+/-}$, heterozygous Mn-SOD deficiency; ROS, reactive oxygen species; VASP, vasodilator stimulated phosphoprotein.

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Abstract

Nitroglycerin (GTN)-induced tolerance was reported to be associated with increased levels of reactive oxygen species (ROS) in mitochondria. In the present study we further investigated the role of ROS for the development of nitrate tolerance by using heterozygous manganese superoxide dismutase knockout mice (Mn-SOD$^{+/-}$). Mn-SOD is acknowledged as a major sink for mitochondrial superoxide. Vasodilator potency of mouse aorta in response to ACh and GTN was assessed by isometric tension studies. Mitochondrial ROS formation was detected by L-012-enhanced chemiluminescence and mitochondrial aldehyde dehydrogenase (ALDH-2) activity was determined by an HPLC-based assay. Aortic rings from Mn-SOD$^{+/-}$ mice showed normal endothelial function and vasodilator responses to GTN. In contrast, preincubation of aorta with GTN or chronic GTN infusion caused a markedly higher degree of tolerance as well as endothelial dysfunction in Mn-SOD$^{+/-}$ as compared to wild type. Basal as well as GTN-stimulated ROS formation was significantly increased in isolated heart mitochondria from Mn-SOD$^{+/-}$ mice correlating well with a marked decrease in ALDH-2 activity in response to *in vitro* and *in vivo* GTN-treatment. The data presented indicate that deficiency in Mn-SOD leads to a higher degree in tolerance and endothelial dysfunction associated with increased mitochondrial ROS production in response to *in vitro* and *in vivo* GTN challenges. These data further point to a crucial role of ALDH-2 in mediating GTN bioactivation as well as development of GTN tolerance and underline the important contribution of ROS to these processes.

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Although organic nitrates such as nitroglycerin (glyceryl trinitrate, GTN) have been used for over a century in the therapy of cardiovascular diseases such as stable and unstable angina (Abrams, 1995) the underlying mechanisms of nitrate bioactivation and development of nitrate tolerance remain elusive. The anti-ischemic effects of organic nitrates are largely due to venous and coronary artery dilation as well as improvement of collateral blood flow, which all decrease myocardial oxygen consumption and are mediated by nitric oxide or a related species. However, the use of organic nitrates is limited due to the rapid development of tolerance and cross-tolerance to endothelium-dependent and independent vasodilators. An impairment of the NO-signaling pathway by increased formation of reactive oxygen species (ROS) (Munzel et al., 1995b) as well as an impaired biotransformation of organic nitrates may contribute to the development of tolerance and cross-tolerance. Recently, the mitochondrial aldehyde dehydrogenase (ALDH-2), which is subjected to an oxidative mechanism-based inactivation, has been identified as a GTN-metabolizing enzyme and a possible important component in the processes leading to tolerance (Chen et al., 2002). Our laboratory further substantiated this concept in an animal model of *in vivo* tolerance and extended previous observations by demonstrating that mitochondria are a major source of ROS formation in response to acute and chronic GTN challenges (Daiber et al., 2004b; Sydow et al., 2004). These results provided indeed the missing link between tolerance and cross-tolerance, the oxidative stress concept and the concept that tolerance is secondary to decreased GTN biotransformation. Since mitochondrial ROS formation seems to play a major role for development of tolerance and cross-tolerance, one could hypothesize that a deficiency in mitochondrial superoxide dismutase (Mn-SOD) would render vascular tissue more susceptible for the development of tolerance.

There are two different types of Mn-SOD deficient mice. Removal of exon 1 and 2 shows lethality at 21 days due to neuronal abnormalities (Lebovitz et al., 1996), whereas

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removal of exon 3 shows lethality at 10 days with dilated ventricular cardiomyopathy (Li et al., 1995). The lack of Mn-SOD causes an increase in mitochondrial superoxide levels, which in turn leads to destruction of iron-sulfur-cluster [4Fe-4S] proteins (Flint et al., 1993). In Mn-SOD$^{-/-}$ mice aconitase activity in the heart is decreased by 42.6 %, which combined with a decrease in succinic acid dehydrogenase activity will impair the citric acid cycle and lead to lipid accumulation in the liver and muscle (Li et al., 1995). The estimation that 1-2 % of all electrons transported by the respiratory chain will ultimately result in the formation of superoxide justifies the importance of Mn-SOD for survival in all mammals (Robinson, 1998). The importance of Mn-SOD was also underlined by the finding that Cu,Zn-SOD overexpression could not compensate for the lack of Mn-SOD: the lethality of these animals was unchanged (Copin et al., 2000). The expression of Mn-SOD, in contrast to Cu,Zn-SOD isoforms (cytosolic and extracellular), can be induced by cytokines (Hennet et al., 1993) and oxidative stress (Shull et al., 1991), but Mn-SOD is also subject to oxidative inactivation, namely nitration and dimerization of essential tyrosine residues. Overexpression of Mn-SOD in mice protected from myocardial ischemia/reperfusion (I/R) injury (Jones et al., 2003), in cells, however, this condition was associated with a hydrogen peroxide-induced up-regulation of matrix-degrading metalloproteinase-1 (Wenk et al., 1999).

This is the first study that uses heterozygous Mn-SOD deficiency (Mn-SOD$^{+/-}$) in mice as a tool to assess the role of oxidative stress for the development of *in vitro* nitrate tolerance and cross-tolerance upon acute GTN treatment of isolated murine aortic rings. The expression of Mn-SOD in Mn-SOD$^{+/-}$ mice is approximately decreased by 50 % as compared to wild type (wt) animals, leading to distinct ultrastructural damage of the myocard, with swelling and disruption of mitochondria and accumulation of lipid droplets, increased nitrotyrosine formation and lipid peroxidation as well as activation of apoptosis signaling pathways in the heart *in vivo* (Strassburger et al., 2005). Cu,Zn-SOD deficiency is well

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characterized with respect to the vascular system and endothelial dysfunction (Didion et al., 2002; Lynch et al., 1997), but there is only little known about vascular consequences of Mn-SOD deficiency. One report presented data that endothelial function (response to acetylcholine) in Mn-SOD$^{+/-}$ mice was not altered in comparison to wt animals (Andresen et al., 2004). With the present studies we sought to focus on the acute GTN-responsiveness as well as on GTN-tolerance development in wt mice and Mn-SOD$^{+/-}$ mice in response to *in vitro* and *in vivo* GTN challenges, and whether ALDH-2 activity and the GTN bioactivation are affected by Mn-SOD deficiency.

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Materials and Methods

Materials. For induction of *in vitro* tolerance and isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). For induction of *in vivo* tolerance, GTN was used from a solution in ethanol (102 g/l) which was obtained from UNIKEM (Copenhagen, Denmark). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dihydroethidium (hydroethidine, DHE) was obtained from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

Animal Model, *In Vitro* and *In Vivo* Nitrate Tolerance. In the present study we used female mice aged 4-8 months on a mixed genetic background (C57Bl/6x129/Ola). Experiments were performed with 16 wt and 16 Mn-SOD$^{+/-}$ mice. Mn-SOD$^{+/-}$ mice were generated according to a published procedure (Strassburger et al., 2005) in the laboratory of K. Scharffetter-Kochanek. Briefly, male mice carrying two SOD2flox alleles were crossed to K14Cre females that, due to Keratin 14 expression in oocytes, also express Cre recombinase in maturing oocytes (Hafner et al., 2004). Since Cre recombinase remains active in oocytes until the paternally inherited SOD2flox allele becomes accessible after fertilization all animals derived from such breedings carry a stably deleted SOD2 allele (SOD2-). SOD2$^{+/-}$ mice were further bred with wt animals of the outbred strain more than 10 times. The absence of the K14Cre allele in the heterozygous offspring was proven by Southern blot analysis. The deletion of exon 3 of the Mn-SOD gene was determined by Southern blot analysis. As exon 3 of the Mn-SOD gene codes for the domain important for tetramer formation of the Mn-SOD, deletion of this domain results in a complete loss of the activity of the enzyme. The

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deficiency of the Mn-SOD activity was determined using a specific activity assay as described recently (Strassburger et al., 2005).

In vitro tolerance development as a result of GTN treatment was assessed by *ex vivo* incubation of murine vessels with 200 µM GTN for 30 min at 37 °C in Krebs-HEPES buffer (composition in g/l: 5.78 NaCl, 0.35 KCl, 0.37 $CaCl_2$, 0.30 $MgSO_4$, 2.1 $NaHCO_3$, 0.14 K_2HPO_4, 5.21 HEPES and 2.0 D-glucose) followed by a 1 h wash-out phase, and subsequent recording of concentration-response curves with GTN. *In vivo* tolerance was induced by chronic infusion of mice with GTN by implanted micro-osmotic pumps (alzet, model 1007D, 0.5 µl/h for 7 d) from Durect Corp. (Cupertino, CA). Infusion of the solvent ethanol served as a control. To determine the infusion rate of GTN that causes tolerance, female wt mice (C57Bl, 5-6 months) were infused with either ethanol or GTN at high (100 µg/h, 220 nmol/min/kg) and low (16 µg/h, 35 nmol/min/kg) dose for 3 d. Based on these results, 4 female wt (C57Bl/6x129/Ola) and 4 female Mn-$SOD^{+/-}$ mice (all 6-8 months) were infused with ethanol and the same number of animals was infused with GTN (16 µg/h, 35 nmol/min/kg) for 4 d. After this period, the animals were sacrificed and aorta as well as hearts were subjected to further analysis. All animals were treated in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Eppendorf and of the University Hospital Mainz.

Isometric tension studies. Vasodilator responses to GTN and ACh were assessed with endothelium-intact isolated murine aortic rings mounted for isometric tension recordings in organ chambers, as described previously (Munzel et al., 1995a). Briefly, mice were anesthetized using CO_2. Thoracic aortas were rapidly removed and cut into ring segments of

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about 3 mm length and mounted in organ chambers for isometric tension recording. Preliminary studies revealed that the optimum resting tension for tone development in response to 80 mM KCl was 1.00 g, which was achieved by gradual stretching over one hour. Following preconstriction with prostaglandine $F_{2\alpha}$ to reach 50 to 80 % of maximal tone induced by KCl, a concentration-response to increasing concentrations of the endothelium-dependent vasodilator acetylcholine (ACh: 1 nM to 3 µM) and the endothelium independent vasodilator nitroglycerin (GTN: 1 nM to 30 µM) was established as described.

Western blot analysis. Aortic segments (1 cm) from wt and Mn-SOD$^{+/-}$ mice were incubated for 5 min with GTN (0.1 µM), frozen and homogenized in liquid nitrogen. The expression of the phosphorylated vasodilator stimulated phosphoprotein (P-VASP) was determined as previously described (Oelze et al., 2000). Immunoblotting was performed with a mouse monoclonal P-VASP phosphoserine 239 antibody (16C2, 1.5 µg/ml; Calbiochem, Schwalbach, Germany). Detection was performed by ECL with peroxidase conjugated anti–rabbit/mouse secondary antibodies (1:10 000, Vector Lab., Burlingame, CA).

ALDH-2 Dehydrogenase and Esterase Activity in Isolated Mouse Heart Mitochondria and Dehydrogenase Activity in Isolated Aortic Segments. The activity of ALDH in isolated mitochondria was determined by measuring the conversion of benzaldehyde to benzoic acid. Mouse heart mitochondria were prepared according to a previously published method (Raha et al., 2000) which was slightly modified (Daiber et al., 2004b). The mitochondrial fraction (total protein approximately 5-10 mg/ml) was kept on ice and diluted to approximately 1 mg/ml protein in 0.25 ml of PBS and preincubated for 30 min at room temperature. In some experiments mitochondria were incubated with GTN (5 or 50 µM) for 30 min prior to ALDH substrate addition. For measurement of ALDH-2 dehydrogenase

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activity, benzaldehyde (400 µM) was added to the mitochondrial suspension and the samples were incubated for another 30 min at 37 °C. For determination of vascular dehydrogenase activity, aortic rings of 3-4 mm length were incubated with benzaldehyde (400 µM) for 30 min at 37 °C. For measurement of ALDH-2 esterase activity, methylbenzoate (1 mM) was added and the samples were incubated for another 30 min at 37 °C. Mitochondrial samples were sonicated, centrifuged at 20,000g (4 °C) for 20 min, and the supernatant was purified by size exclusion centrifugation through a Microcon YM-10 filter device from Millipore (Bedford, USA). 200 µl of each sample was subjected to HPLC analysis. The details were recently published (Daiber et al., 2004b).

Measurement of Reactive Oxygen Species Production from Isolated Heart Mitochondria and from Isolated Aortic Segments. Mitochondrial stock solutions were diluted to final total protein concentrations of approximately 0.1 mg/ml in 0.5 ml PBS. The dye L-012 (100 µM) was used as described (Daiber et al., 2004a) to quantify ROS following addition of the complex II substrate succinate (4 mM final concentration). Chemiluminescence was monitored over 5 min using a Lumat LB9507 from Berthold Techn. (Bad Wildbad, Germany) and the signal at 5 min was expressed as counts/min. ROS production was quantified in mitochondria from wt and Mn-SOD$^{+/-}$ mice in the presence or absence of GTN (50 µM) or antimycin A (20 µg/ml). Vascular ROS production was qualitatively detected by DHE (0.1 µM)-derived fluorescence in aortic tissue sections as previously described (Hink et al., 2001) and by L-012 (100 µM)-derived chemiluminescence from isolated aortic rings (length 3-4 mm) in Krebs-HEPES buffer (composition as described above). Chemiluminescence was monitored over 20 min using a Lumat LB9507 and the signal at 20 min expressed as counts/min.

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Statistical Analysis. Results are expressed as mean±SEM. One-way ANOVA (with Bonferroni's or Dunn's correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, L-012-derived chemiluminescence, ALDH-2 dehydrogenase as well as esterase activity and protein expression. The EC_{50} value for each experiment was obtained by log-transformation. Significance is indicated by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ and respective other symbols.

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Results

Vasodilator responses. The basal response to ACh was almost identical in both, wt and Mn-SOD$^{+/-}$ mice (Fig. 1A and Table 1). Upon pre-treatment of vessels with 200 µM GTN, those from Mn-SOD$^{+/-}$ mice showed a highly significant degree of endothelial dysfunction (cross-tolerance to ACh) which was absent in those from wt mice. The efficacy of ACh in Mn-SOD$^{+/-}$ aorta was dramatically changed upon GTN pre-treatment (maximal relaxation 52±4 % vs. 69±4 % in untreated wt tissue). Similar results were obtained for the response to GTN (Fig. 1B and Table 1). The Mn-SOD$^{+/-}$ aorta showed a decrease in maximal relaxation as compared to wt aorta. Both groups showed a significant degree of tolerance upon pre-treatment with 200 µM GTN which was significantly more pronounced in aortas from Mn-SOD$^{+/-}$ mice (Fig. 1B and Table 1).

To assess the role of Mn-SOD deficiency on development of *in vivo* tolerance, mice were chronically infused with GTN. In a preceding experiment, wt mice were treated with high and low dose of GTN or the solvent alone to determine the infusion rate of GTN which is required to induce tolerance in mice. As determined by isometric tension studies with aortic rings in organ baths, the low dose of GTN (16 µg/h) induced neither nitrate tolerance nor cross-tolerance with a tendency to slightly shift the ACh and GTN dose-response curve to the right side (Fig. 1C, 1D and Table 2). In contrast, the high dose of GTN (100 µg/h) induced a marked degree of nitrate tolerance and cross-tolerance as envisaged by the right-shifted dose-response curves to ACh and GTN and significantly decreased efficacy of both vasodilators as well as a significantly reduced potency of GTN (Fig. 1C, 1D and Table 2).

To test the GTN-induced NO-signaling we performed Western blots to determine the phosphorylation state of VASP, a ubiquitous substrate of the cGMP-dependent protein kinase (cGK-I). No significant difference in P-VASP levels between ethanol and GTN (16 µg/h) *in vivo* infusion of wt mice was observed, whereas P-VASP expression was significantly

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decreased in GTN infused Mn-SOD$^{+/-}$ mice as compared to the ethanol controls, respectively (Fig. 1E). P-VASP levels in both Mn-SOD-deficient groups were significantly decreased as compared to the wt groups, indicating an impaired GTN bioactivation and/or NO-signaling in the deficient animals.

Mitochondrial and Vascular Reactive Oxygen Species Formation. The formation of mitochondrial ROS was detected by a chemiluminescence (CL)-based assay using the luminol analogue L-012. Isolated murine heart mitochondria were assessed for basal ROS production, and for ROS production in the presence of GTN and antimycin A. ROS formation was significantly increased by 80 % in Mn-SOD$^{+/-}$ mitochondria as compared to wt mitochondria (56,802±3,919 vs. 31,368±2,871 counts/min, see Fig. 2A). In the presence of 50 µM GTN the CL signal in Mn-SOD$^{+/-}$ mitochondria increased by 100 % and that in wt mitochondria by 150 % (113,611±9,736 vs. 78,616±5,071 counts/min). The presence of antimycin A, which preferentially induces generation of mitochondrial superoxide, significantly increased the CL signal in Mn-SOD$^{+/-}$ mitochondria by 58 %, whereas the CL signal in wt mitochondria increased by 88 % (89,863±9,550 in +/- vs. 58,972±5,192 counts/min in wt) (Fig. 2A). Mitochondrial ROS were also detected in isolated heart mitochondria from *in vivo* ethanol or GTN (16 µg/h) treated wt and Mn-SOD$^{+/-}$ mice. GTN infusion had no effect on mitochondrial ROS production of wt mice, whereas it significantly increased that in Mn-SOD$^{+/-}$ mice (Fig. 2B). *In vitro* challenges of isolated mitochondria from *in vivo* treated animals with GTN (25 µM) elevated ROS formation in both groups but the absolute increase was higher in Mn-SOD-deficient mice (Fig. 2B). The ROS-induced signals in ethanol, GTN (16 µg/h) *in vivo* and GTN *in vivo* plus *in vitro* treated Mn-SOD$^{+/-}$ mice were significantly higher than those in similarly treated wt mice indicating the increased basal oxidative stress in deficient animals (Fig. 2B). Differences in the signal intensities

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between Fig. 2A and 2B may be due to chronic ethanol infusion in the second set of experiments.

Vascular ROS formation was detected by DHE-derived fluorescence and L-012-derived chemiluminescence. Vascular ROS production from isolated aortic segments of wt mice was not significantly altered upon infusion with GTN (16 µg/h) *in vivo* as compared to the ethanol treated controls (Fig. 2C). Similarly, vascular ROS formation in ethanol infused deficient mice was not significantly changed as compared to the wt animals, but GTN *in vivo* infusion elevated the signal significantly as compared to the wt treatment groups (Fig. 2C). Fig. 2D shows representative DHE stainings of tissue sections from wt and Mn-SOD$^{+/-}$ aorta. The staining of Mn-SOD$^{+/-}$ material is more intense as compared to wt material, indicating an increased basal production of vascular superoxide in Mn-SOD$^{+/-}$ mice.

Effects of Mn-SOD Deficiency on Mitochondrial ALDH-2 Dehydrogenase, Esterase Activity and Vascular ALDH Dehydrogenase Activity. Basal enzyme activities were not significantly altered in Mn-SOD$^{+/-}$ compared to wt mitochondria (Fig. 3A and 3B). The addition of 5 µM GTN attenuated the dehydrogenase activity in both groups. The decrease amounted to 31 % in Mn-SOD$^{+/-}$ mitochondria and to only 21 % in those from wt mice (decrease from 13.3±0.8 to 9.2±0.8 µM and 14.8±0.8 to 11.7±0.9 µM, respectively) (Fig. 3A). In contrast, the esterase activity in wt mitochondria was almost unaffected by pre-treatment with 50 µM GTN (43.6±4.9 vs. 38.5±6.9 µM), whereas esterase activity in Mn-SOD$^{+/-}$ mitochondria was significantly decreased by 32 % in the presence of GTN (45.3±4.9 vs. 30.8±3.4 µM) (Fig. 3B).

Vascular dehydrogenase activity in isolated aortic segments from ethanol infused deficient mice was not significant different as compared to similarly treated wt mice (Fig. 3C). GTN (16 µg/h) *in vivo* infusion caused no significant decrease of ALDH activity as

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compared to ethanol controls of the same animal group, but GTN infused Mn-SOD$^{+/-}$ mice showed a significantly lower vascular ALDH activity as compared to ethanol treated wt mice (Fig. 3C). Chronic infusion with either ethanol or an ethanolic solution of GTN increased mitochondrial ALDH-2 activity significantly ($p<0.001$ for wt vs. wt/EtOH) in comparison with non-infused animals and ALDH-2 activity rather increased in deficient mice (compare Fig. 3D to 3A). *In vivo* GTN treatment decreased ALDH-2 dehydrogenase activity in both animal groups, the decrease being significantly stronger in deficient mice (Fig. 3D).

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Discussion

Previously, we (Sydow et al., 2004) and others (Chen et al., 2002; de la Lande et al., 2004b; Kollau et al., 2004; Zhang et al., 2004) have shown that ALDH-2 biotransforms GTN *in vitro* and *in vivo* and that inhibition of this enzyme markedly decreases the vasodilator potency of GTN. So far there was only one study questioning the contribution of ALDH-2 to GTN bio-activation (DiFabio et al., 2003). The loss of ALDH-2 activity was associated with or secondary to mitochondrial ROS formation upon chronic or acute challenges to GTN *in vitro* and *in vivo* (Daiber et al., 2004b; Sydow et al., 2004). With the present studies we can demonstrate for the first time that increased oxidative stress within mitochondria from mice with heterozygous Mn-SOD deficiency (Mn-SOD$^{+/-}$) prones vascular tissue to develop tolerance as well as cross-tolerance (endothelial dysfunction) in response to *in vitro* and *in vivo* GTN challenges. These results point to a crucial role of ROS within mitochondria in determining vascular GTN biotransformation and vascular responsiveness to endothelium dependent and independent nitrovasodilators respectively.

Importantly, the degree of tolerance and cross-tolerance was markedly higher in vessels from Mn-SOD$^{+/-}$ vs. wt (Fig. 1A and B, Table 1 and 2). Moreover, basal mitochondrial and vascular ROS formation as well as ROS production in response to *in vitro* and *in vivo* challenges of GTN and the complex III inhibitor antimycin A was substantially increased in Mn-SOD$^{+/-}$ animals (Fig. 2). As a consequence the redox-sensitive ALDH-2 was found to be inhibited upon *in vitro* and *in vivo* challenges with GTN and this effect was more obvious in Mn-SOD$^{+/-}$ mice as compared to wt animals (Fig. 3). In particular, the ALDH-2 esterase activity, which has been proposed to be crucial for GTN bio-activation (Chen et al., 2002) was strikingly more susceptible to GTN-mediated inactivation in Mn-SOD deficient mice (Fig. 3).

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Heterozygous Mn-SOD deficiency does not affect NO-signaling/endothelial function but makes vessels more susceptible to nitrate tolerance and cross-tolerance. The vasodilator potency and efficacy in response to the endothelium-dependent vasodilator ACh was not significantly different in Mn-SOD$^{+/-}$ as compared to wild type mice. This observation was in agreement with recent reports from the group of Heistad that neither the basal response to ACh was changed in Mn-SOD deficient mice nor the ACh response upon treatment with the complex III inhibitor antimycin A, which probably would yield mitochondrial ROS (Andresen et al., 2004). This is even more surprising since DHE staining clearly demonstrated increased ROS production throughout the vascular wall. Interestingly, however, upon pre-incubation of the vessels with GTN (200 µM) there was a quite marked loss of ACh efficacy in the Mn-SOD$^{+/-}$ mice, which was absent in the wt animals, indicating that GTN treatment – presumably by inducing oxidative stress in mitochondria (Daiber et al., 2004b; Sydow et al., 2004) – influenced the ACh response and therefore caused cross-tolerance (endothelial dysfunction) in Mn-SOD deficient mice (Fig. 1A and Table 1).

Interestingly, we could not detect significantly decreased responsiveness to the endothelium independent nitrovasodilator GTN in vessels from Mn-SOD-deficient mice as compared to wt controls. Upon pre-incubation of the isolated vessels from wt and Mn-SOD$^{+/-}$ with GTN (200 µM), however, there was a significant loss of GTN potency in both animal groups being more pronounced in the Mn-SOD$^{+/-}$ mice (Fig. 1B and Table 1). There was no significant difference in the efficacy of GTN pre-treated Mn-SOD$^{+/-}$ and wt vessels (Table 1). These observations were further supported by the effects of *in vivo* GTN (16 µg/h, 35 nmol/min/kg) infusion in deficient mice on GTN-dependent NO-signaling (measured by P-VASP levels, Fig. 1E). This indicates that Mn-SOD deficiency makes the vessels more susceptible to *in vitro* and *in vivo* nitrate tolerance. To address whether this phenomenon is

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related to increased oxidative stress in Mn-SOD$^{+/-}$ mice, we measured superoxide production in mitochondria from wt and Mn-SOD$^{+/-}$ in the presence and absence of GTN.

Heterozygous Mn-SOD deficiency increases basal, GTN- and antimycin A-triggered mitochondrial as well as vascular ROS formation which may be a key-event for the development of nitrate tolerance and cross-tolerance. Oxidative stress plays an important role in the development of nitrate tolerance and cross-tolerance (Hink et al., 2003; Munzel et al., 1995b; Schwemmer and Bassenge, 2003). We have previously identified superoxide and/or peroxynitrite as the reactive species formed in tolerant vessels. In addition, scavengers of peroxynitrite and derived free radicals such as ebselen and uric acid normalized luminol-enhanced chemiluminescence in vessels from GTN treated animals, restored the activity of the cGMP-dependent kinase I and subsequently improved GTN tolerance in isolated rings (Hink et al., 2003). Increased vascular peroxynitrite formation also lead to increased protein tyrosine nitration of the prostacyclin synthase and was associated with an almost complete inhibition of vascular PGI_2 formation in the setting of tolerance (Hink et al., 2003; Warnholtz et al., 2002). Nitration of prostacyclin synthase is a specific footprint of peroxynitrite *in vivo* formation (Zou et al., 1999).

Here, we provide further evidence that a considerably part of the organic nitrate-induced oxidative stress may originate secondary to mitochondrial nitrate metabolism. Using L-012-dependent chemiluminescence (Daiber et al., 2004a) we detected peroxynitrite and superoxide in isolated rat heart mitochondria under basal conditions as well as upon *in vitro* and *in vivo* administration of GTN or antimycin A. As expected, basal mitochondrial ROS formation was significantly increased in mitochondria from Mn-SOD$^{+/-}$ mice as compared to the wt group (Fig. 2A and B). As expected, the absolute increase upon stimulation with bolus or chronic GTN as well as antimycin A was significantly more pronounced in mitochondria

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from Mn-SOD$^{+/-}$ deficient animals (Fig. 2A and B). This indeed indicates that the antioxidant defense system in Mn-SOD deficient mitochondria is impaired and ROS formation is increased under basal conditions. Vascular ROS formation was detected by two methods (DHE-dependent oxidative fluorescent microtopography and L-012-derived chemiluminescence) and also showed that basal as well as GTN-triggered vascular ROS formation was increased in tissue from Mn-SOD$^{+/-}$ mice (Fig. 2C and D).

Heterozygous Mn-SOD deficiency decreases ALDH-2 dehydrogenase and esterase activity in response to GTN and thereby impairs the mitochondrial GTN bio-activation leading to tolerance and endothelial dysfunction. The mitochondrial isoform of ALDH (ALDH-2) was previously identified as a GTN bio-activating enzyme (Chen et al., 2002). We recently demonstrated that ALDH-2 is sensitive to oxidative inactivation by different organic nitrates as well as by oxidants such as hydrogen peroxide or peroxynitrite (Daiber et al., 2004b). ALDH-2 contains three cysteine residues in the catalytic center, rendering the dehydrogenase activity highly sensitive towards oxidative inactivation (Senior and Tsai, 1990; Tsai and Senior, 1991). In addition to its dehydrogenase activity, ALDH-2 also exhibits esterase activity, which has been proposed to be essential for the bio-activation of GTN (Chen et al., 2002). This activity also involves oxidation-sensitive cysteine residues (Tsai and Senior, 1991). As a consequence of increased oxidative stress in the Mn-SOD$^{+/-}$ mitochondria we found that ALDH-2 dehydrogenase activity and esterase activity was significantly decreased in mitochondria but also isolated aortic rings upon *in vitro* or *in vivo* treatment with GTN (Fig. 3).

Mechanistic implications. Mn-SOD together with glutathione peroxidase is the most important antioxidant defense enzyme in mitochondria. Complete deficiency is disastrous for

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the organism and causes death within 2-3 weeks (Lebovitz et al., 1996; Li et al., 1995). We here demonstrate that heterozygous Mn-SOD deficiency increases the basal but also the GTN and Antimycin A-induced formation of mitochondrial ROS. As a consequence of GTN-induced ROS production the GTN bio-activating enzyme ALDH-2 (Daiber et al., 2004b) or its repair system which may involve mitochondrial lipoic acid stores and/or the glutathione/glutathione reductase system (unpublished observation) will be impaired. Inactivation of ALDH-2 will subsequently slow down the mitochondrial bioactivation of GTN which will be manifested by the phenomenon of nitrate tolerance and cross-tolerance and further point to a crucial role of this enzyme in the bioactivation process of GTN. A hypothetical unifying scheme is shown in Fig. 4. With respect to the importance of oxidative stress for the development of nitrate tolerance it is worth to mention that not all organic nitrates induce oxidative stress. Less potent nitrates such as isosorbide dinitrate (ISDN) and isosorbide-5-mononitrate (ISMN) will probably generate less mitochondrial ROS as compared to GTN (Daiber et al., 2004b). Also for the highly potent pentaerythritol tetranitrate (PETN) studies have shown that this nitrate does neither induce oxidative stress nor nitrate tolerance (Jurt et al., 2001). This is because of intrinsic antioxidative responses triggered by PETN, such as increased expression of the protective proteins heme oxygenase-1 and ferritin (Oberle et al., 2003).

Although GTN-triggered ROS formation within mitochondria explains the tolerance phenomenon due to impaired GTN biotransformation, it is difficult to understand why this process should also cause the phenomenon of endothelial dysfunction, since ROS formed within mitochondria will not easily cross the mitochondrial membrane. It is possible, however, that GTN-derived-ROS (superoxide and peroxynitrite) might react with iron-sulfur-cluster proteins disrupting the respiratory chain (Flint et al., 1993). Therefore, it seems conceivable to conclude that GTN initiates a vicious cycle of mitochondrial ROS formation

which could further be exaggerated by oxidative inactivation of Mn-SOD (MacMillan-Crow et al., 1996). Some of these ROS might escape the mitochondrial space and impair NO-signaling by direct reaction with NO or by an oxidative inactivation of soluble guanylyl cyclase (Brune et al., 1990; Mulsch et al., 1997). Alternatively, GTN-triggered mitochondrial ROS might lead to opening of mitochondrial ATP-dependent potassium channels (Zhang et al., 2001) and thereby trigger further ROS production (Lebuffe et al., 2003). The exact components of this molecular cascade are still not well determined, and this latter hypothesis remains rather speculative. However, it is interesting to note that mitochondrial ROS production and subsequent K-ATP channel opening might determine both GTN-induced protective (preconditioning-mimetic) effect (Dawn and Bolli, 2002) and, upon chronic GTN treatment, increased oxidative damage (Munzel et al., 1995b) leading to tolerance and endothelial dysfunction. Finally, we would like to emphasize that nitrate tolerance and cross-tolerance are probably multi-factorial phenomena and also other processes contribute to the degree of tolerance as demonstrated by the marked effects of endothelium denudation of tolerant vessels on GTN responsiveness (de la Lande et al., 2004a; Munzel et al., 1995b). With respect to the “oxidative stress concept” there are also other sources of ROS that may trigger the development of tolerance such as GTN-activated NADPH oxidases (Munzel et al., 1995a; Schwemmer and Bassenge, 2003) and probably an uncoupled NO-synthase.

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Footnotes

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Legends to Figures

Fig. 1. Effect of heterozygous Mn-SOD deficiency on vasodilator activity of ACh and GTN in murine aorta and on development of *in vitro* and *in vivo* tolerance upon acute and chronic GTN challenges. Dose-response curves to ACh (**A**) or GTN (**B**) in wt aorta (closed circles), GTN (200 µM) *in vitro* treated wt aorta (closed triangles), Mn-SOD$^{+/-}$ aorta (open inverted triangles) and GTN (200 µM) *in vitro* treated Mn-SOD$^{+/-}$ aorta (open squares) were recorded upon preconstriction with prostaglandin $F_{2\alpha}$. Data represent the mean ± SEM of 10-12 (GTN-treated) or 17-18 (untreated) independent experiments. Dose-response curves to ACh (**C**) or GTN (**D**) in aorta from ethanol *in vivo* treated (circles), GTN (16 µg/h) *in vivo* treated (triangles) or GTN (100 µg/h) *in vivo* treated wt mice (squares) were recorded upon preconstriction with prostaglandin $F_{2\alpha}$. Data represent the mean ± SEM of 12-14 (ethanol treated), 5-7 (GTN (16 µg/h) treated) or 9-11 (GTN (100 µg/h) treated) independent experiments. For statistical analysis see Table 1 and 2. (**E**) P-VASP expression was determined as a measure of GTN (0.1 µM)-elicited cGMP-dependent protein kinase (cGK-I) activation (NO-signaling) in ethanol or GTN (16 µg/h) *in vivo* infused wt and Mn-SOD$^{+/-}$ mice. Data represent the mean ± SEM of 4 independent experiments and a representative blot.

Fig. 2. Effect of heterozygous Mn-SOD deficiency on basal, antimycin A- and GTN (*in vitro* and *in vivo*)-stimulated mitochondrial ROS formation. (**A**) The formation of mitochondrial ROS was detected using L-012 (100 µM)-derived chemiluminescence (CL) in mitochondria isolated from murine hearts. Mitochondrial suspensions (0.2 mg/ml final protein) were treated with either GTN or antimycin A (AA). The measurements were initiated by adding succinate (4 mM). Data represent the mean ± SEM of 32-33 (basal), 18-19 (GTN)

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or 6 (AA) independent experiments. Mitochondrial (**B**) and vascular (**C**) ROS were also detected in isolated heart mitochondria from *in vivo* ethanol or GTN (16 µg/h) treated wt and $MnSOD^{+/-}$ mice. The effects of acute GTN (25 µM) challenges on mitochondrial ROS were also tested. The chemiluminescence signal (counts/min) of vascular ROS formation was normalized on dry weight (mg) of aortic segments. Data represent the mean ± SEM of 3 independent experiments. (**D**) The basal formation of ROS was detected by microscopy using DHE (0.1 µM)-derived fluorescence in aortic tissue sections from wt or $Mn\text{-}SOD^{+/-}$ mice. The auto-fluorescence of the lamina is stained in green and scale bars represent 100 µM. Images shown were recorded at 20-fold magnification and are representative for 3 (wt) or 4 ($Mn\text{-}SOD^{+/-}$) animals.

Fig. 3. Effect of heterozygous Mn-SOD deficiency on basal ALDH-2 activity and GTN-dependent inhibition of ALDH-2. The activities of ALDH-2 were determined by HPLC-based measurements of the conversion of benzaldehyde (**A**, dehydrogenase activity) or methylbenzoate (**B**, esterase activity) to benzoic acid in suspensions of mouse heart mitochondria (from wt or $Mn\text{-}SOD^{+/-}$ mice) which were treated or not treated with GTN (5 or 50 µM). Data represent the mean ± SEM of 15-20 (dehydrogenase) or 13-16 (esterase) independent experiments. Vascular (**C**) and mitochondrial (**D**) dehydrogenase activity was also measured in aortic segments from *in vivo* ethanol or GTN (16 µg/h) treated wt or $Mn\text{-}SOD^{+/-}$ mice. Vascular dehydrogenase activity was normalized on dry weight (mg) of aortic segments. Data represent the mean ± SEM of 4 (aorta) and 7 (mitochondria) independent experiments.

Fig. 4. Hypothetical scheme illustrating the mechanisms underlying oxidative stress-dependent development of nitrate tolerance in response to GTN treatment. Under

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normal conditions GTN (as well as pentaerythritol tetranitrate (PETN) and its trinitrate metabolite (PETriN)) is bioactivated by mitochondrial ALDH yielding 1,2-glyceryl dinitrate and nitrite that undergoes further reduction (by the mitochondrial respiratory chain or acidic disproportionation) and finally yields a vasodilator (either NO, S-nitrosothiol or a related species). ALDH-2 is inactivated during bioactivation of GTN by oxidation of essential thiol groups to the disulfide. ALDH-2 reductase activity is restored by reduced lipoic acid, the oxidized form is reduced by the glutathione (GSH)-glutathione reductase system (GR/NADPH). Chronic GTN treatment induces mitochondrial reactive oxygen and nitrogen species formation (ROS/RNS). The diffusion-limited reaction of nitric oxide and superoxide yields peroxynitrite ($ONOO^-$). This oxidative stress may inhibit GTN bioactivation by inactivation of ALDH-2 or inhibition of the ALDH-2 repair system including lipoic acid as well as the glutathione/glutathione reductase system. Moreover, superoxide may decrease the bioavailability of the vasodilator released from GTN bioactivation. Mn-SOD and glutathione peroxidase (GPx) will protect the bioactivation system from oxidative damage by breakdown of superoxide and hydrogen peroxide. Mn-SOD may prevent the formation of peroxynitrite, which is a highly toxic compound to the respiratory chain at different sites of mitochondrial respiration.

Table 1. EC_{50} values and maximal relaxation of aorta with or without acute *in vitro* challenges from wt and Mn-SOD$^{+/-}$ mice in response to ACh and GTN.

	Potency, EC_{50} (-log M) [b]		Efficacy, % Maximal relaxation [c]	
Group [a]	ACh	GTN	ACh	GTN
wt	7.24 ± 0.13 (n=18)	7.18 ± 0.05 (n=18)	66.48 ± 3.45 (n=18)	81.48 ± 1.95 (n=18)
wt - *in vitro* GTN	7.17 ± 0.31 (n=10)	7.10 ± 0.07 (n=12)	64.12 ± 4.82 (n=10)	70.05 ± 2.15§§ (n=12)
Mn-SOD$^{+/-}$	7.23 ± 0.16 (n=17)	7.13 ± 0.07 (n=16)	69.02 ± 3.98 (n=17)	77.15 ± 2.64 (n=16)
Mn-SOD$^{+/-}$ - *in vitro* GTN	7.25 ± 0.11 (n=10)	6.82 ± 0.13* (n=12)	51.99 ± 4.02* (n=10)	58.74 ± 3.32$^{\#\#\#,\$}$ (n=12)

[a] *in vitro* GTN indicates that vessels in these groups were incubated for 30 min in the presence of 200 µM GTN, followed by a 1 h washout period prior to isometric tension measurement.

[b] EC_{50}-values were normalized on maximal relaxation. * indicates significance (p = 0.017) vs. Mn-SOD$^{+/-}$ group.

[c] * indicates significance (p = 0.036) vs. Mn-SOD$^{+/-}$ group, §§ indicates significance (p = 0.00187) vs. wt group, $^{\#\#\#}$ indicates significance (p = 0.00052) vs. Mn-SOD$^{+/-}$ group and $^{\$}$ indicates significance (p = 0.0413) vs. wt - *in vitro* GTN group.

Table 2. EC_{50} values and maximal relaxation in response to ACh and GTN of aorta from wt mice treated *in vivo* with GTN or sham (ethanol)

	Potency, EC_{50} (-log M) [b]		Efficacy, % Maximal relaxation [b]	
Group	ACh	GTN	ACh	GTN
wt – *in vivo* ethanol [a]	7.10 ± 0.07 (n=14)	7,22 ± 0.04 (n=12)	71.38 ± 2.97 (n=14)	82.17 ± 2.38 (n=12)
wt – *in vivo* GTN (16 µg/h) [a]	7.09 ± 0.13 (n=7)	7.03 ± 0.09 (n=5)	64.92 ± 3.32 (n=7)	84.61 ± 2.56 (n=5)
wt – *in vivo* GTN (100 µg/h) [a]	6.95 ± 0.11 (n=11)	6.43 ± 0.15***,# (n=9)	54.59 ± 3.97*** (n=11)	74.96 ± 3.07**,## (n=9)

[a] separate set of experiments in which relaxation of aorta from *in vivo* ethanol or GTN (low and high dose) treated wt mice was assessed.

[b] EC_{50}-values were normalized on maximal relaxation. ** indicates significance ($p<0.01$) vs. wt – *in vivo* ethanol [a] group (*** means $p<0.005$), # indicates significance ($p<0.05$) vs. wt – *in vivo* GTN (16 µg/h) group (## means $p<0.01$).

Figure 1A

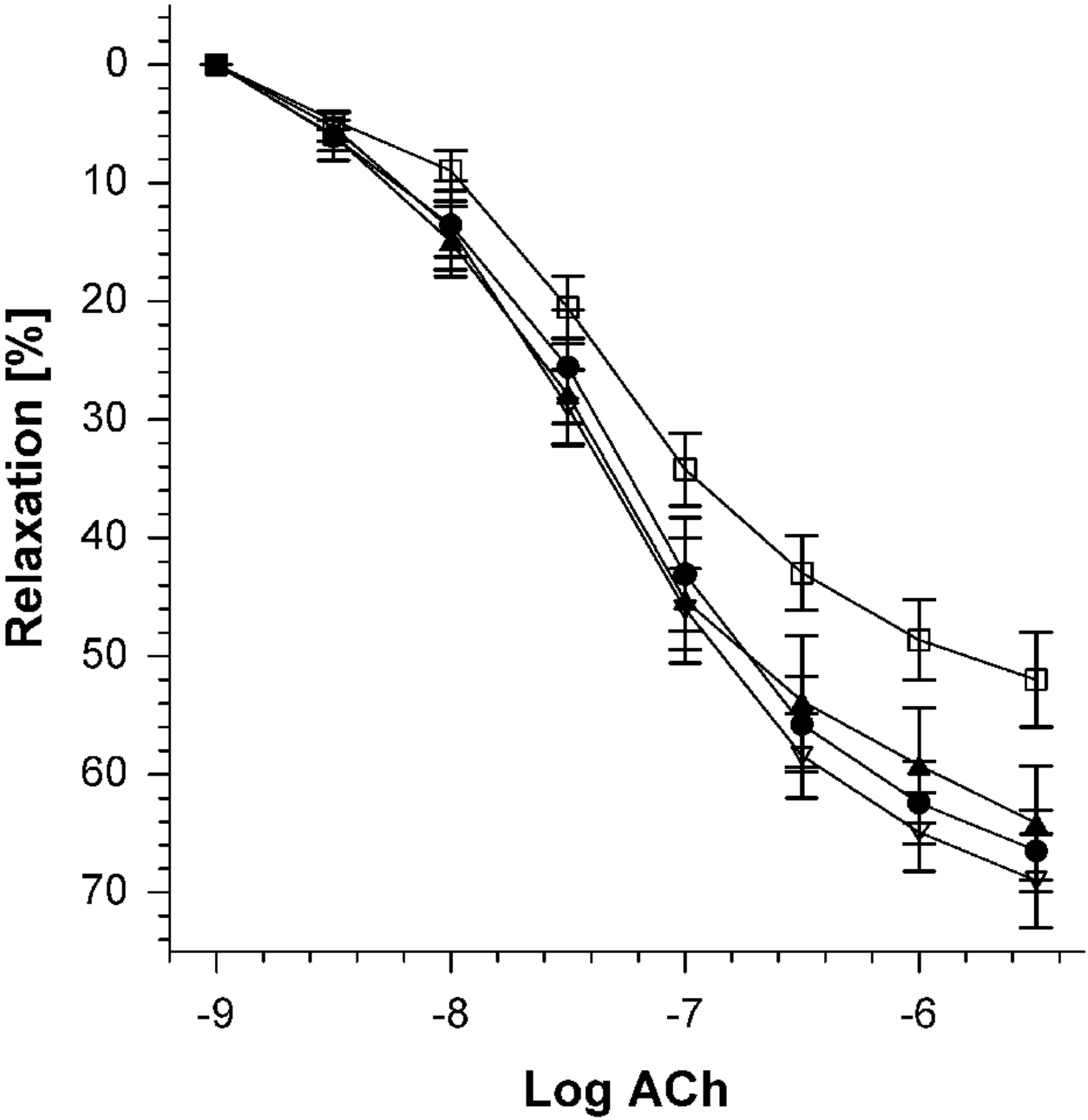


Figure 1B

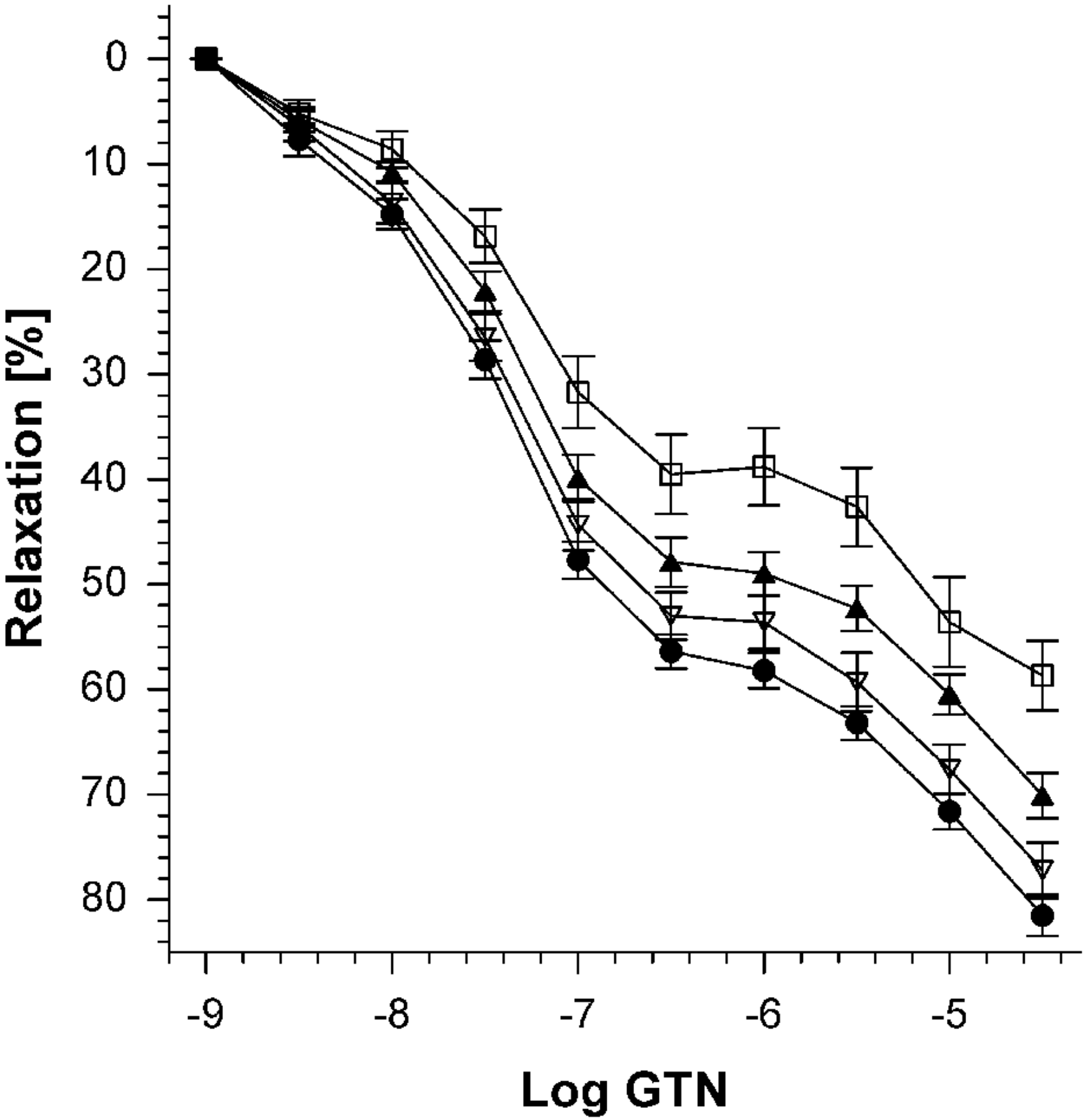


Figure 1C

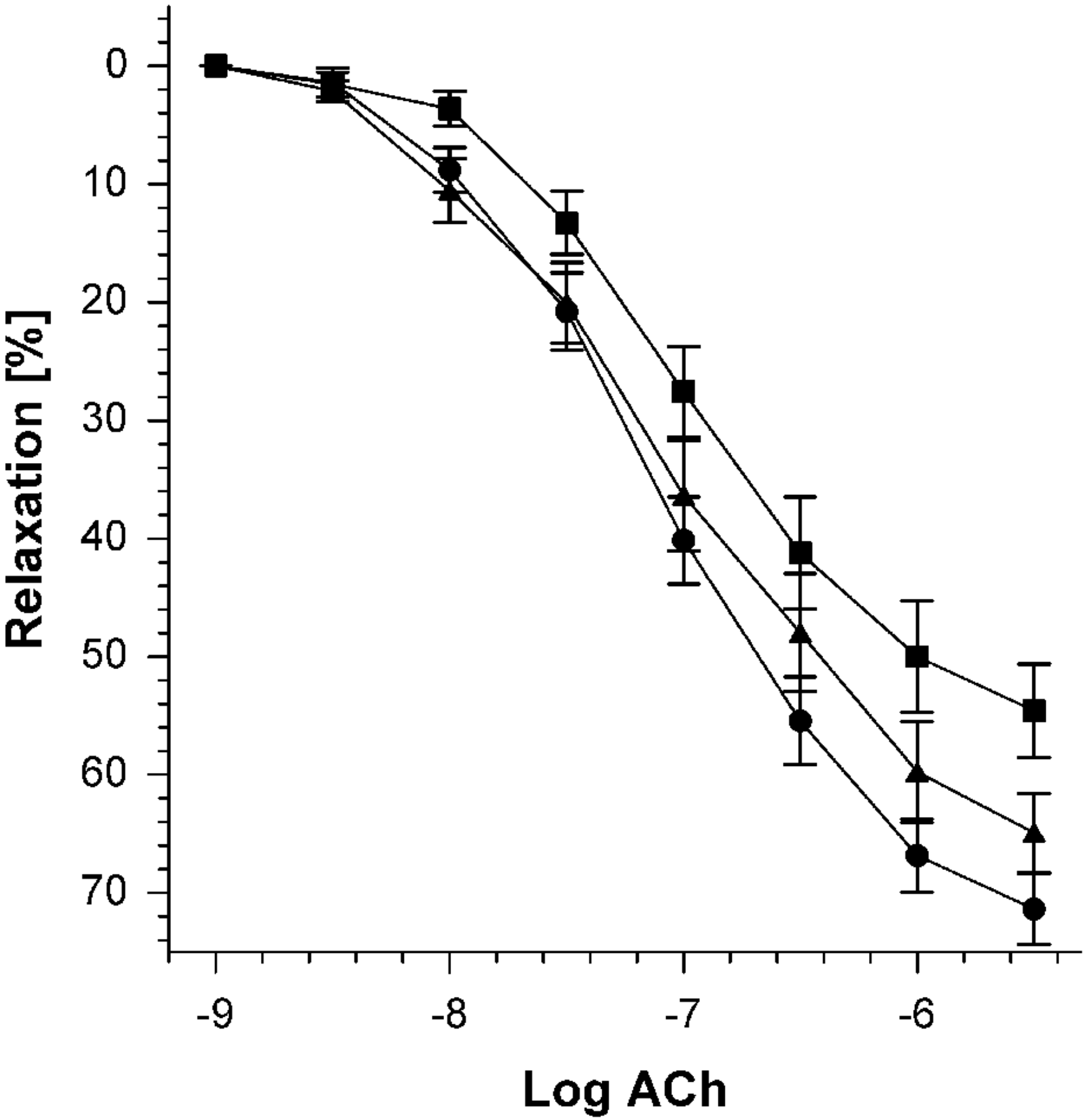


Figure 1D

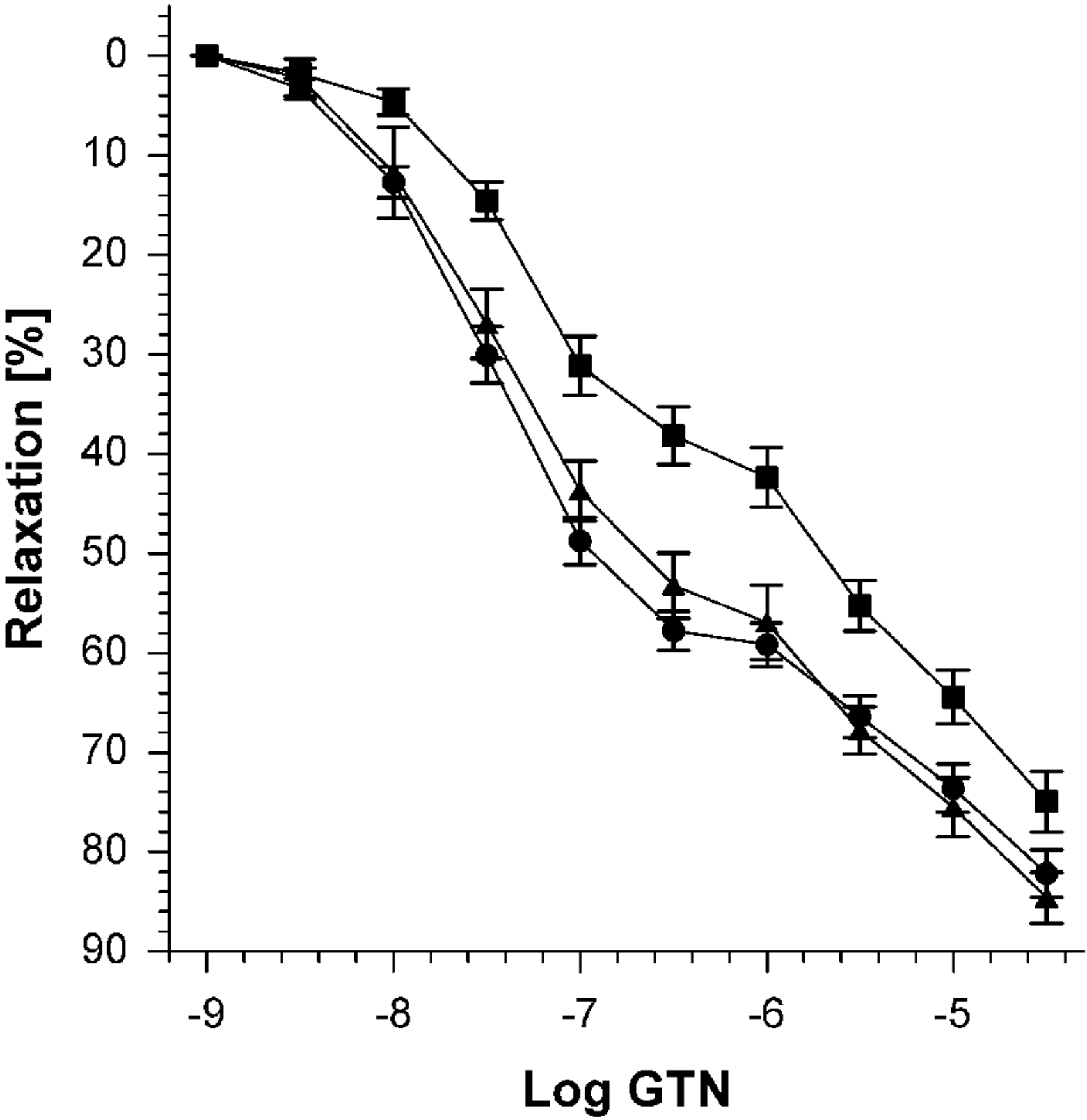


Figure 1E

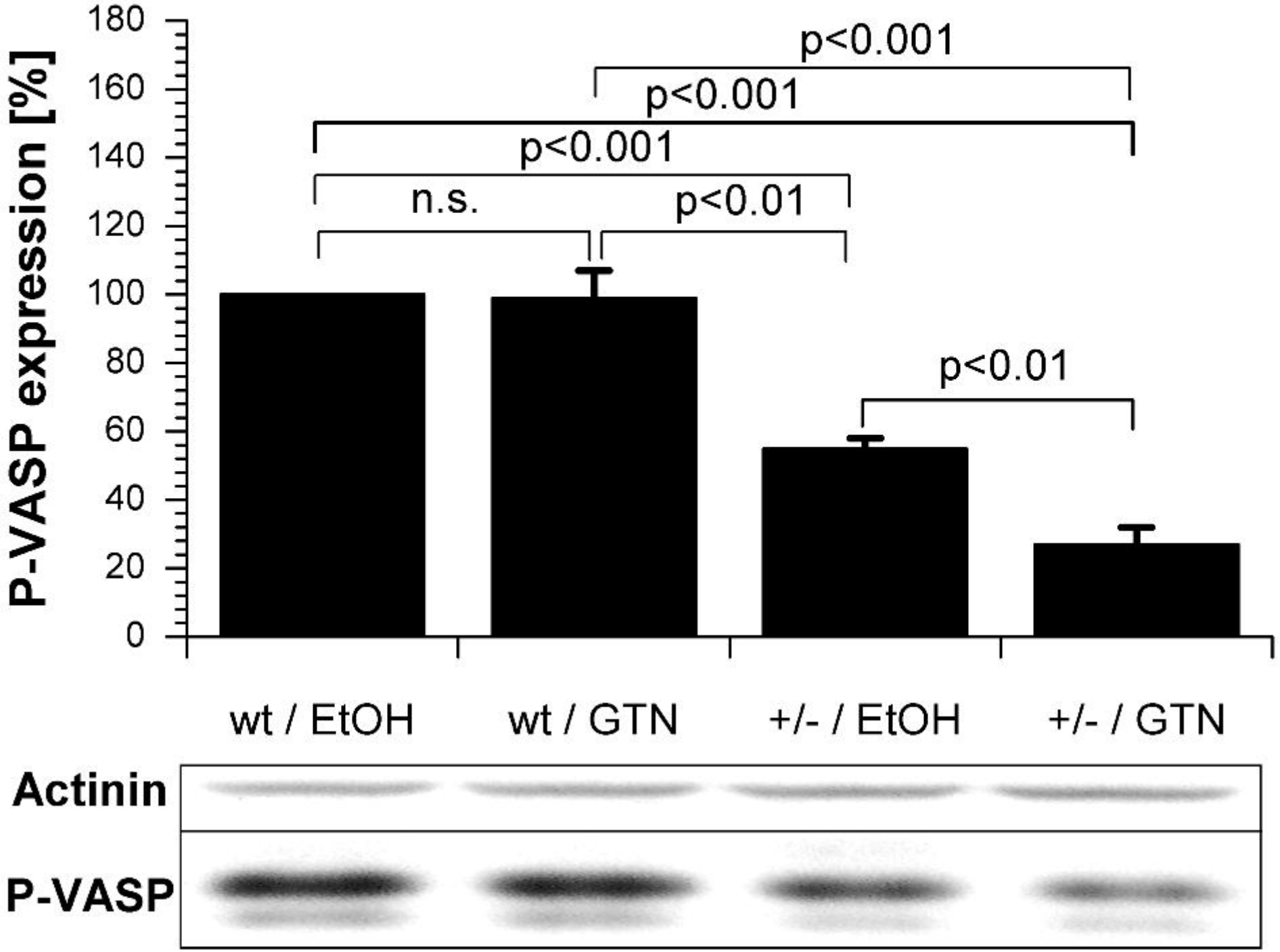

P-VASP expression [%]
180
160
140
120
100
80
60
40
20
0
p<0.001
p<0.001
p<0.001
n.s.
p<0.01
p<0.01
wt / EtOH
wt / GTN
+/- / EtOH
+/- / GTN
Actinin
P-VASP

Figure 2A

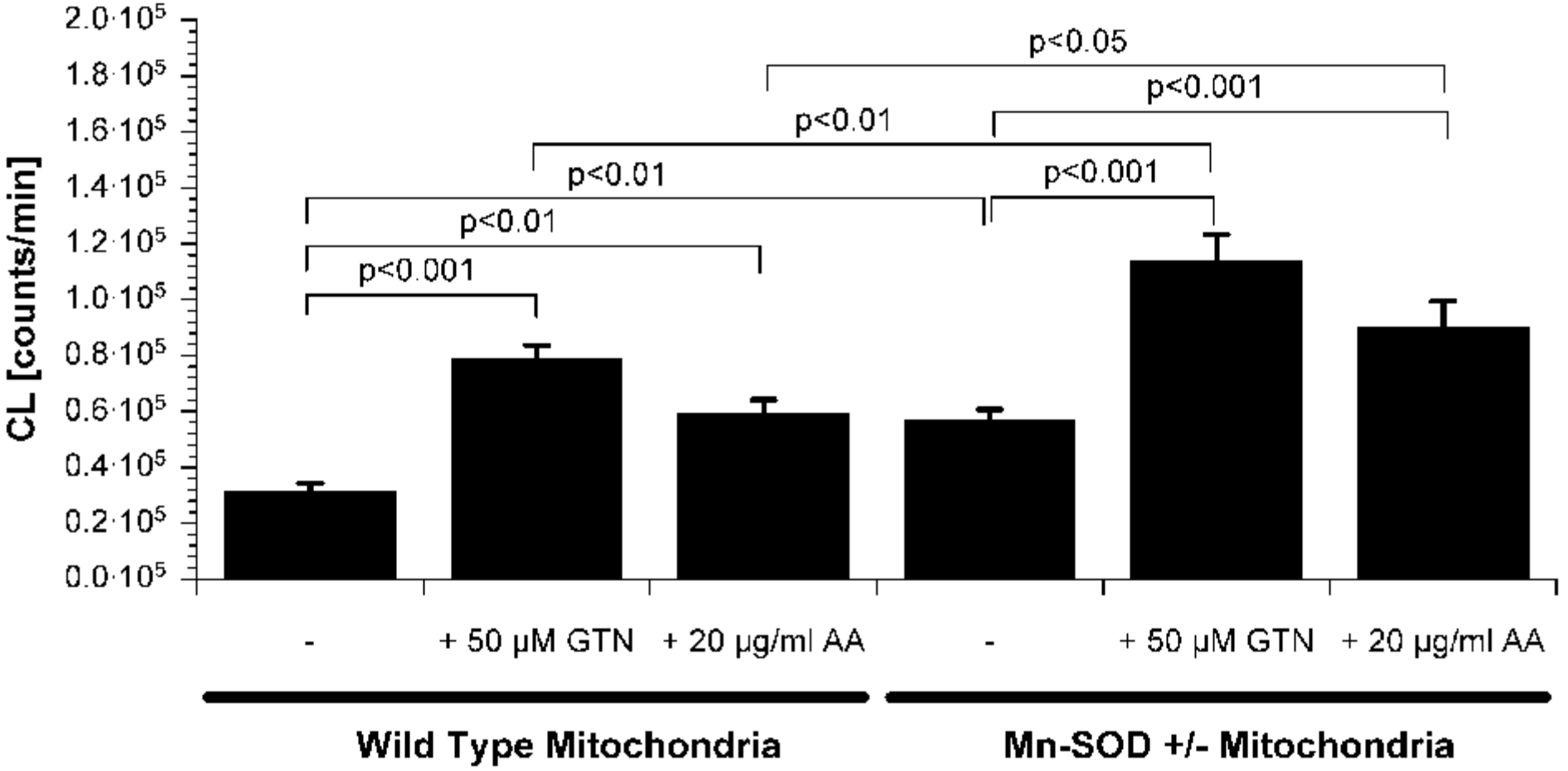

CL [counts/min]
2.0·10^5
1.8·10^5
1.6·10^5
1.4·10^5
1.2·10^5
1.0·10^5
0.8·10^5
0.6·10^5
0.4·10^5
0.2·10^5
0.0·10^5
p<0.05
p<0.001
p<0.01
p<0.01
p<0.001
p<0.01
p<0.001
-
+ 50 µM GTN
+ 20 µg/ml AA
-
+ 50 µM GTN
+ 20 µg/ml AA
Wild Type Mitochondria
Mn-SOD +/- Mitochondria

Figure 2B

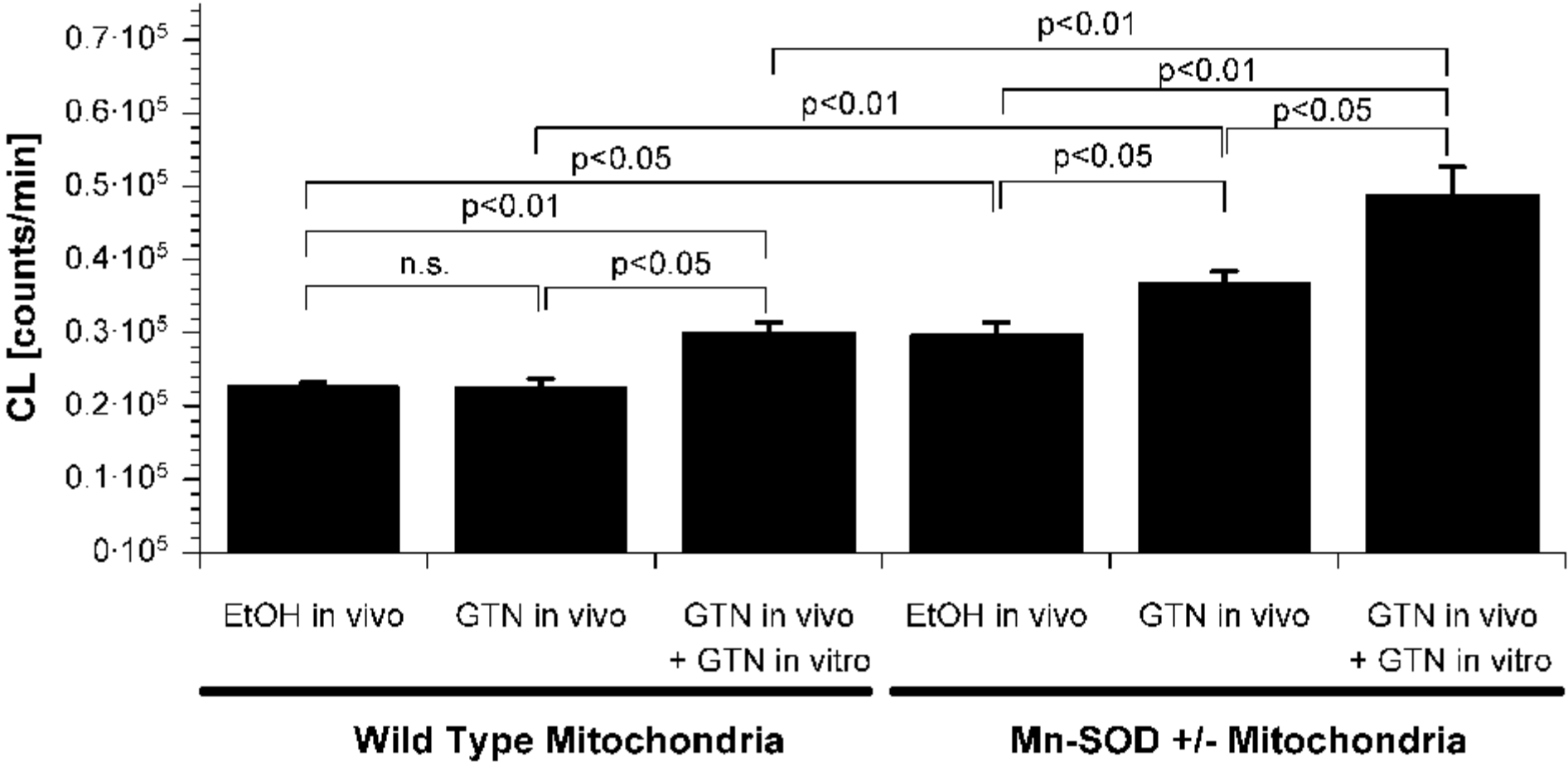

CL [counts/min]
0.7·10^5
0.6·10^5
0.5·10^5
0.4·10^5
0.3·10^5
0.2·10^5
0.1·10^5
0·10^5
p<0.01
p<0.01
p<0.01
p<0.05
p<0.05
p<0.05
p<0.01
n.s.
p<0.05
EtOH in vivo
GTN in vivo
GTN in vivo + GTN in vitro
EtOH in vivo
GTN in vivo
GTN in vivo + GTN in vitro
Wild Type Mitochondria
Mn-SOD +/- Mitochondria

Figure 2C

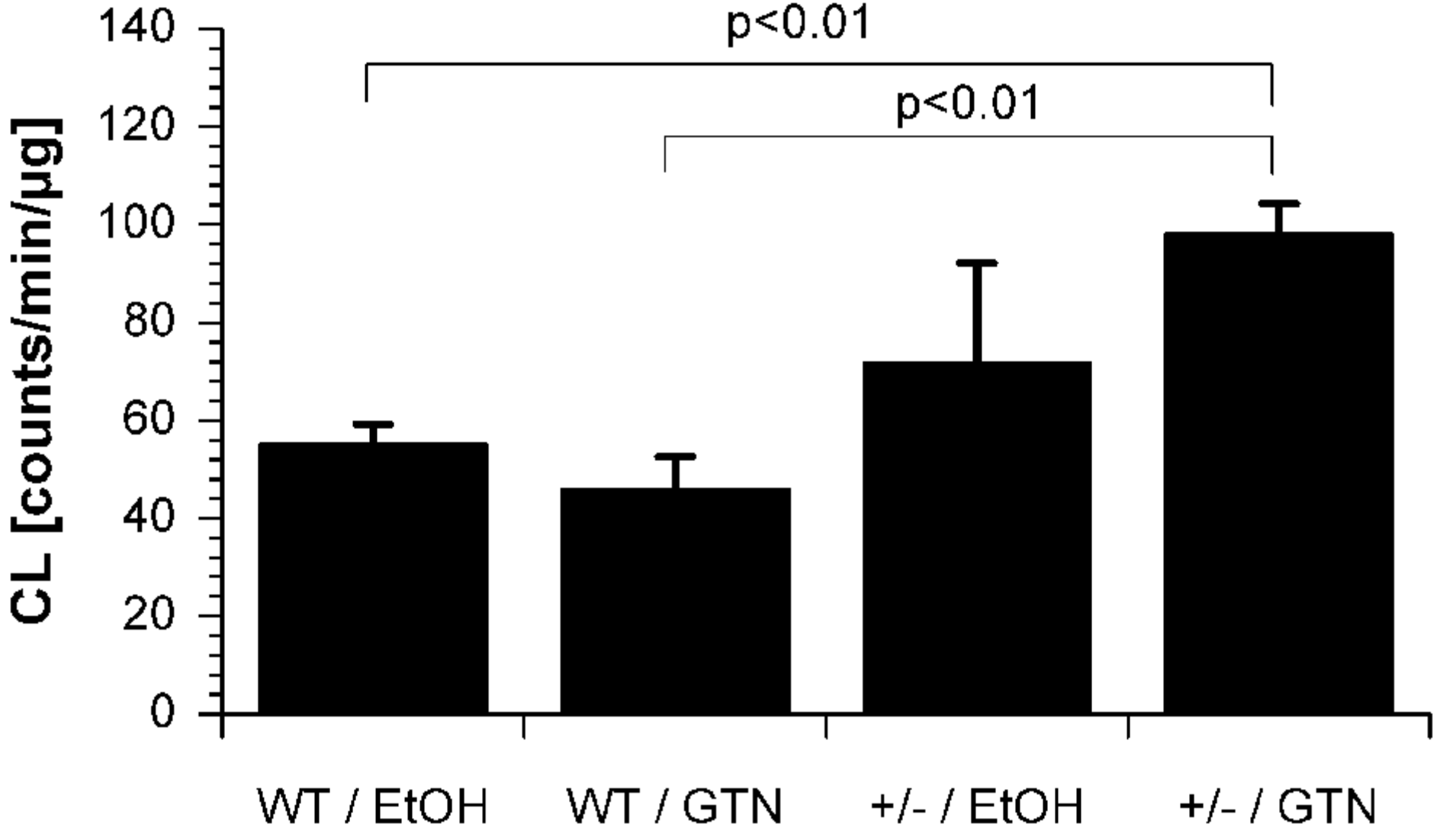

p<0.01
p<0.01
CL [counts/min/µg]
140
120
100
80
60
40
20
0
WT / EtOH
WT / GTN
+/- / EtOH
+/- / GTN

Figure 2D

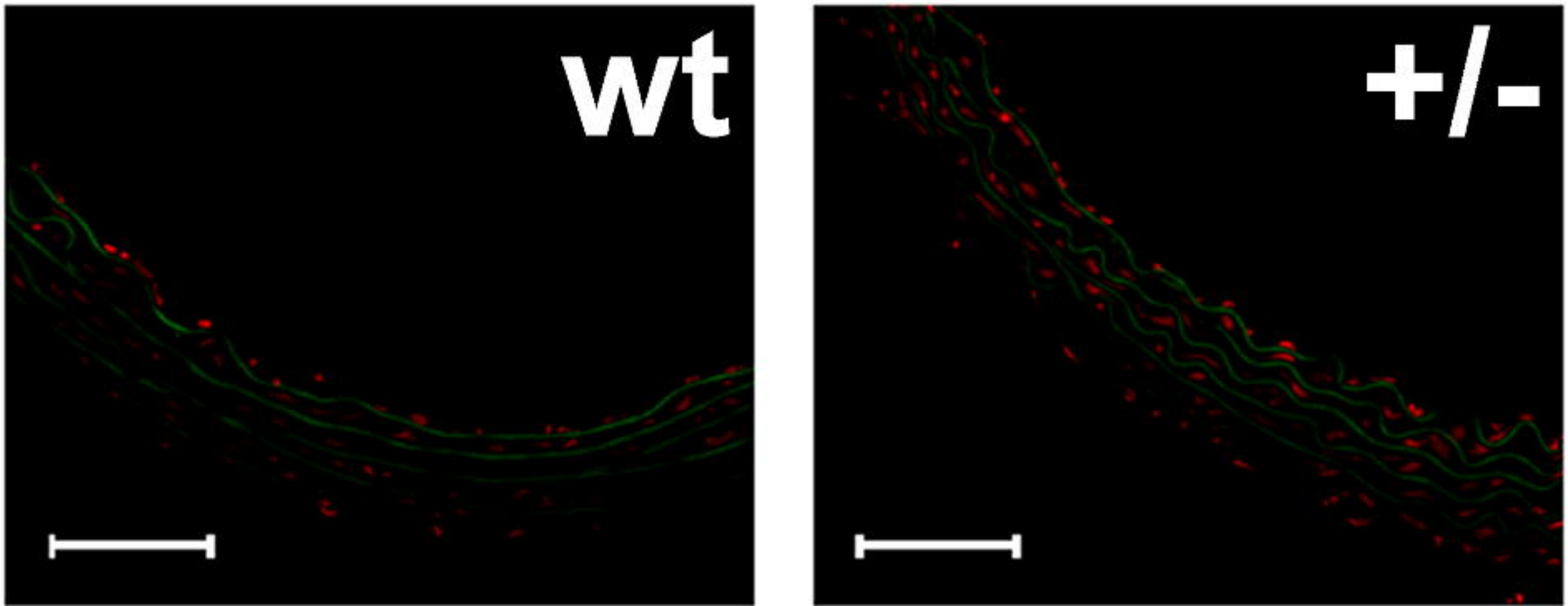


Figure 3A

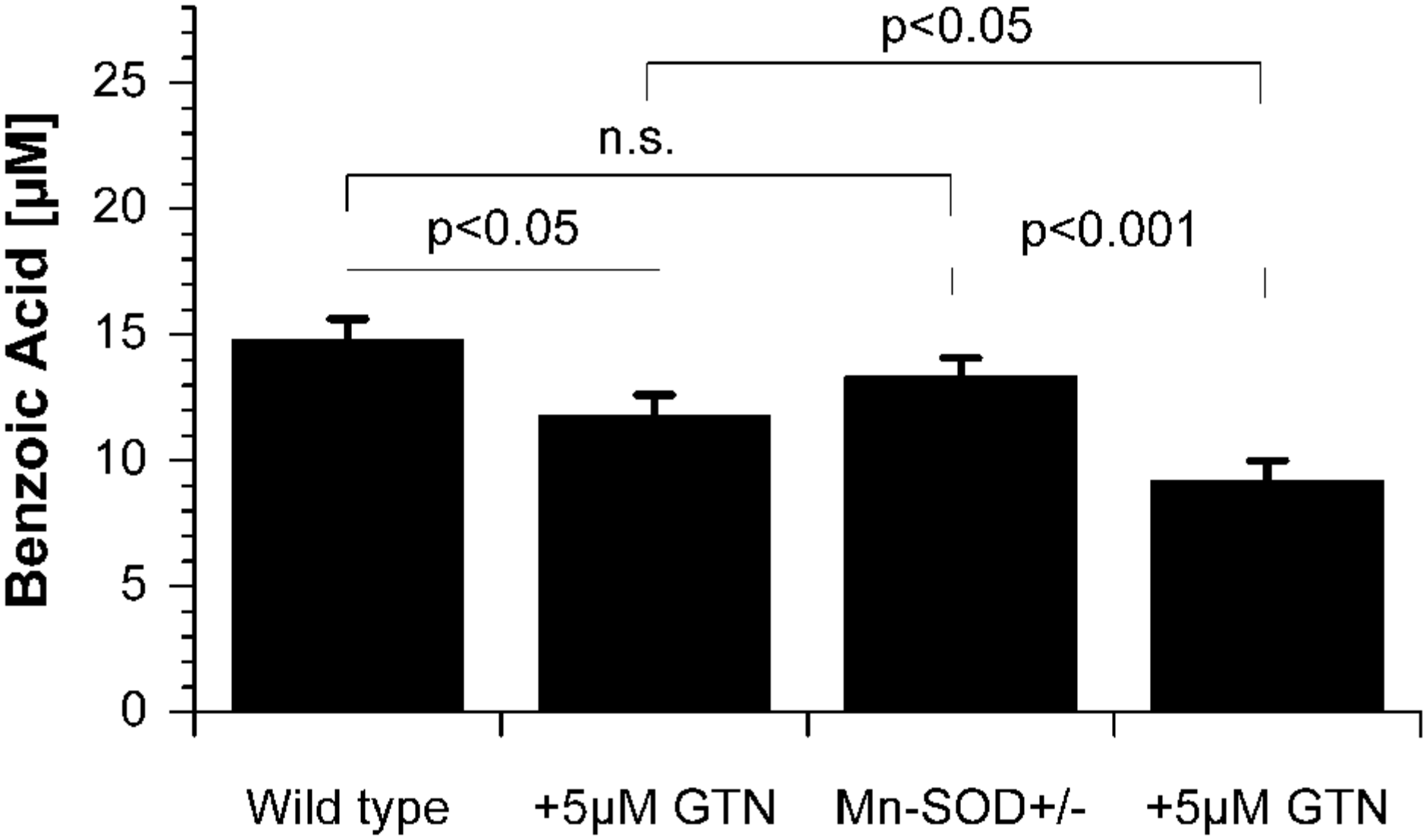

p<0.05
n.s.
p<0.05
p<0.001
Benzoic Acid [µM]
25
20
15
10
5
0
Wild type
+5µM GTN
Mn-SOD+/-
+5µM GTN

Figure 3B

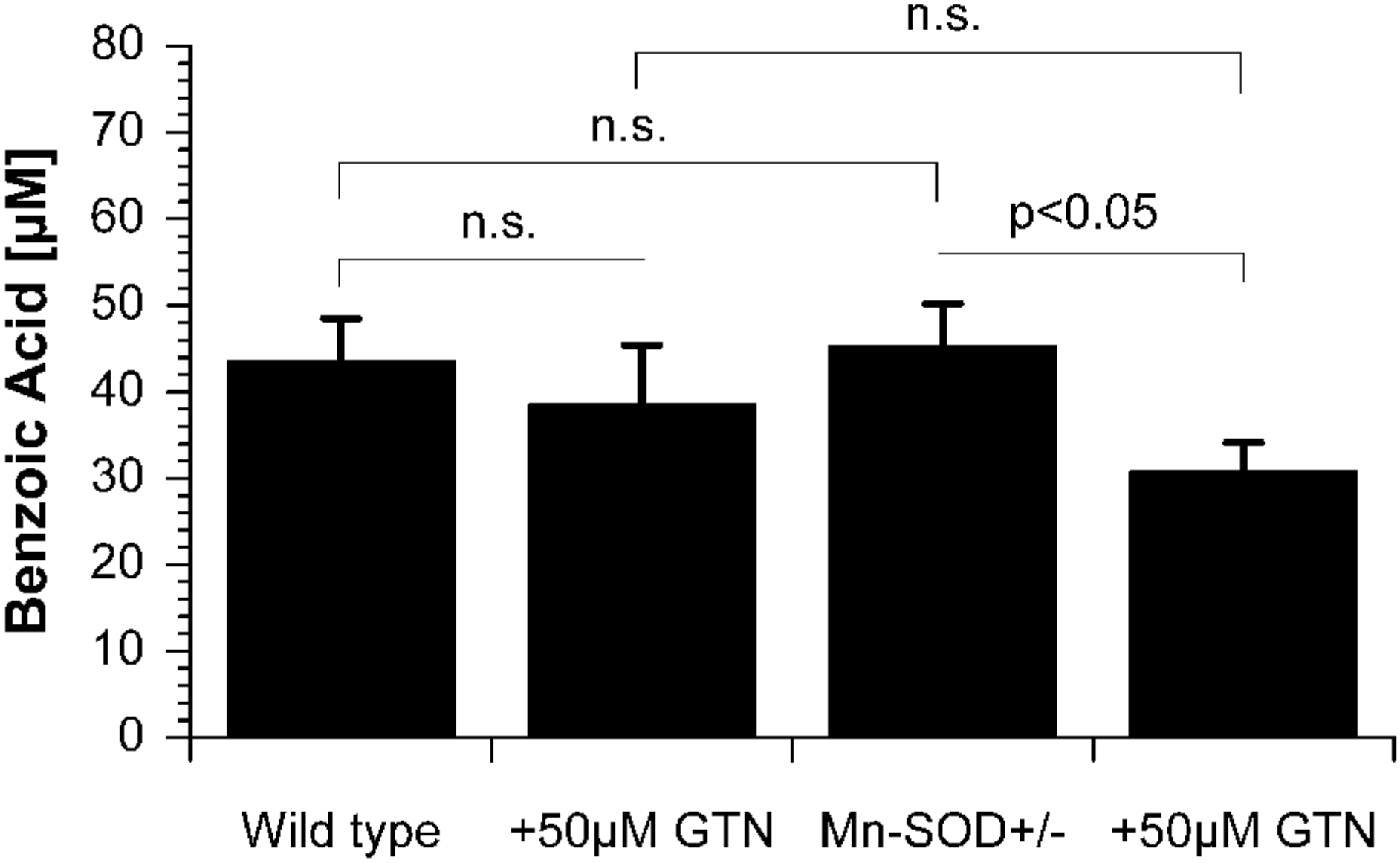

n.s.
n.s.
n.s.
p<0.05
Benzoic Acid [µM]
80
70
60
50
40
30
20
10
0
Wild type
+50µM GTN
Mn-SOD+/-
+50µM GTN

Figure 3C

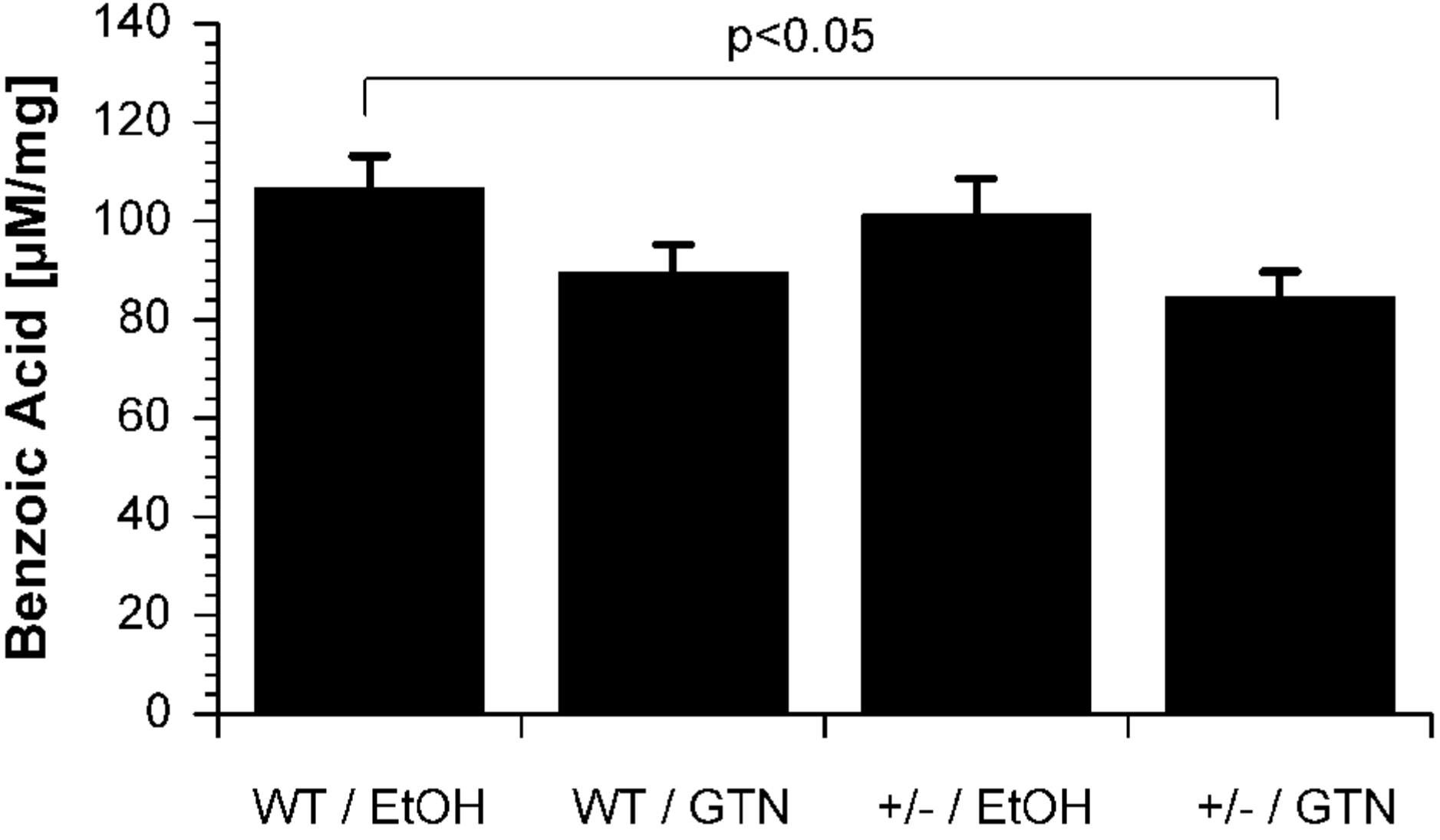

p<0.05
Benzoic Acid [µM/mg]
140
120
100
80
60
40
20
0
WT / EtOH
WT / GTN
+/- / EtOH
+/- / GTN

Figure 3D

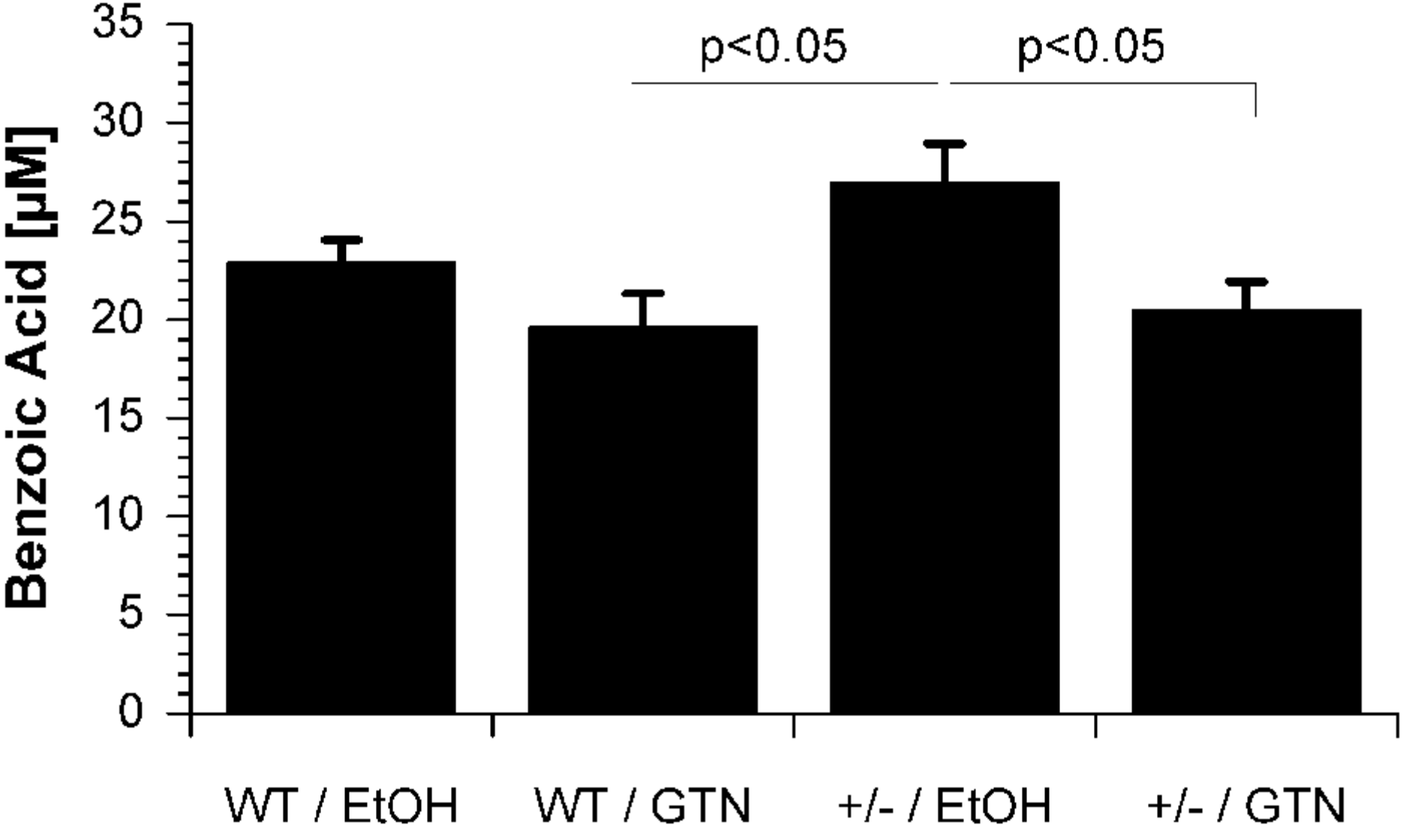
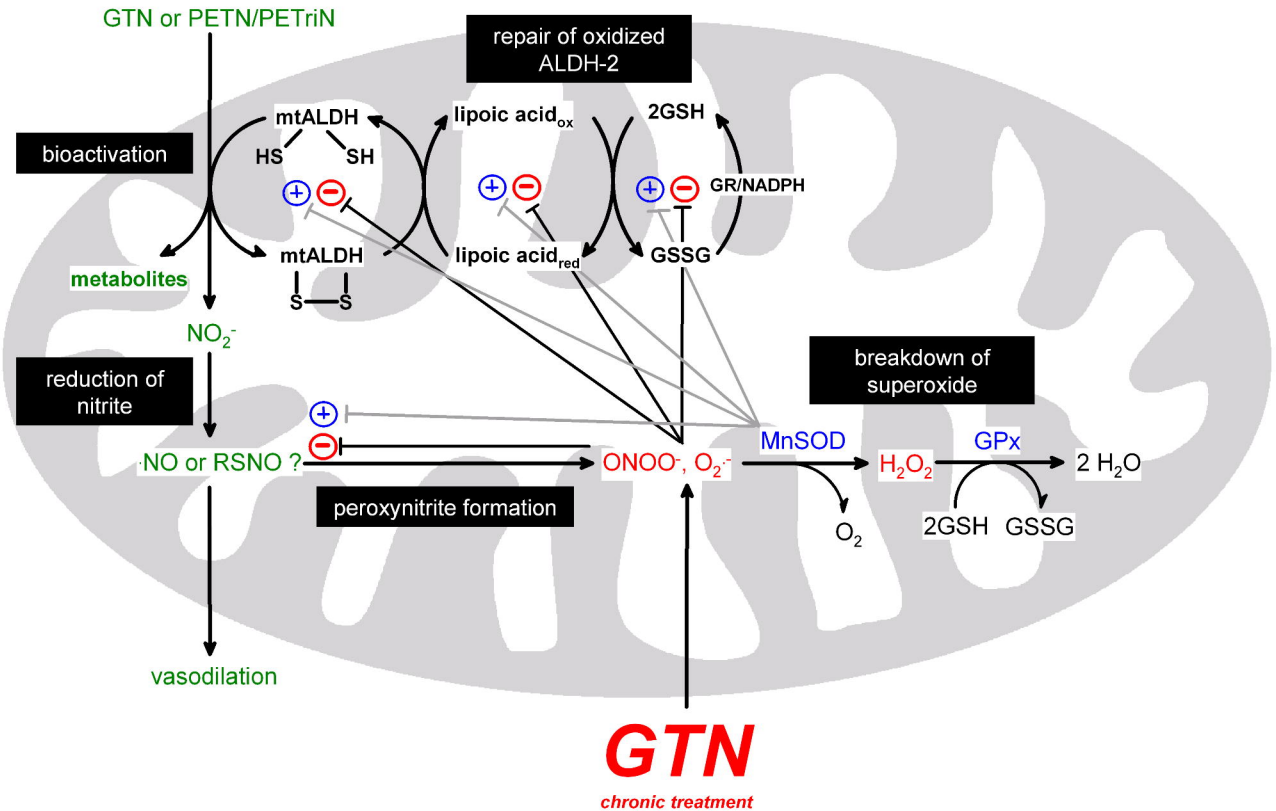

35
30
25
20
15
10
5
0
Benzoic Acid [µM]
p<0.05
p<0.05
WT / EtOH
WT / GTN
+/- / EtOH
+/- / GTN

Figure 4


GTN or PETN/PETriN
repair of oxidized ALDH-2
bioactivation
mtALDH
HS
SH
lipoic acid$_{ox}$
2GSH
GR/NADPH
mtALDH
S—S
lipoic acid$_{red}$
GSSG
metabolites
NO_2^-
reduction of nitrite
breakdown of superoxide
NO or RSNO ?
peroxynitrite formation
$ONOO^-$, $O_2^{\cdot-}$
MnSOD
H_2O_2
GPx
2 H_2O
O_2
2GSH
GSSG
vasodilation
GTN
chronic treatment