Online Supplement for Dikalova et al.

HPLC measurement of cellular and aortic $O_2^{\bullet-}$: Superoxide was measured using dihydroethidium (DHE) and an HPLC-based assay. Cultured cells were washed with Krebs/HEPES buffer and incubated with 10 μM DHE for 20 min at 37°C. Aortic sections were incubated with 50 μM DHE for 30 min at 37°C. Media was removed and cells or tissue were transferred to methanol for extraction of superoxide-specific product 2-hydroxyethidium (2-OH-E^+) and kept at -80°C. Separation of ethidium, 2-OH-E^+ and dihydroethidium was performed using a Beckman HPLC System Gold model with a C-18 reverse phase column (Nucleosil 250, 4.5 mm; Sigma-Aldrich).

Dose-dependent angiotensin II stimulation of endothelial $O_2^{\bullet-}$: Cellular $O_2^{\bullet-}$ production was measured in HAEC following 4 hours of treatment with 0, 40, 100 or 200 nM angiotensin II using HPLC and DHE (Online Figure I). Angiotensin II increased $O_2^{\bullet-}$ production in a dose-dependent manner with maximum stimulation at 200 nM concentration.

Effect of mitoTEMPO and mitoTEMPO-H on xanthine oxidase: Superoxide was generated using xanthine oxidase and xanthine to measure SOD activity and to investigate SOD-scavenging properties of mitoTEMPO and mitoTEMPO-H. Additional experiments were performed to exclude a potential effect of these agents on xanthine oxidase activity. Neither mitoTEMPO nor mitoTEMPO-H affected xanthine oxidase activity as reflected by the accumulation of urate (Online Figure II).

HPLC analysis of superoxide specific product of mitoSOX: MitoSOX can produce both superoxide specific (2-OH-Mito-E^+) and non-specific (Mito-E^+) fluorescent products [1]. To provide validation of $O_2^{\bullet-}$ detection with mitoSOX we used HPLC to quantify 2-OH-Mito-E^+ and Mito-E^+ as was previously described [1]. Incubation of mitoSOX with xanthine oxidase and xanthine resulted in the specific accumulation of 2-OH-Mito-E^+ which was inhibited by superoxide dismutase (Online Figure IIIA). Control or angiotensin II stimulated HAEC were supplemented with 4 μM mitoSOX for 20 minutes and then washed with Krebs/HEPES buffer. The cells were then harvest in methanol for extraction of 2-OH-Mito-E^+ and Mito-E^+. Analysis of cellular extracts showed accumulation of both 2-OH-Mito-E^+ and Mito-E^+. Stimulation of cells with angiotensin II (200 nM for 4 hours) increased levels of 2-OH-Mito-E^+ but did not change Mito-E^+ (Online Figure IIIB). Supplementation with mitoTEMPO (25 nM for 15 minutes) before addition of mitoSOX inhibited accumulation of 2-OH-Mito-E^+ (Online Figure IIIC). These data support the specificity of $O_2^{\bullet-}$ measurements with mitoSOX.

Site-specific detection of cellular and mitochondrial $O_2^{\bullet-}$ by DHE and mitoSOX: In order to validate $O_2^{\bullet-}$ detection by DHE and mitoSOX we have performed HPLC measurements of $O_2^{\bullet-}$ specific

products 2-OH-E^+ and 2-OH-Mito-E^+ [1] in HAEC treated with NADPH oxidase activator phorbol myristate acetate (PMA, 10 μM) or mitochondrial complex III inhibitor antimycin A (AA, 1 μM). HPLC analysis showed that stimulation of $O_2^{\bullet-}$ production in cytoplasm with PMA was reflected in accumulation of 2-OH-E^+ in DHE supplemented cells but did not result in significant accumulation of 2-OH-Mito-E^+ in mitoSOX supplemented cells (Online Figure IV). Antimycin A induced increase in mitochondrial $O_2^{\bullet-}$ was reflected in accumulation of 2-OH-Mito-E^+ in mitoSOX supplemented cells but did not raised the level of 2-OH-E^+ in DHE supplemented cells (Online Figure IV). These data demonstrate site-specific detection of cellular and mitochondrial $O_2^{\bullet-}$ by DHE and mitoSOX.

Investigation of direct effect of mitoTEMPO on NADPH oxidase activity: To perform these studies, we stimulated BAEC with angiotensin II (200 nM for 4 hours) and measured NADPH oxidase activity in membrane fractions using ESR as described in the Methods section. Angiotensin II significantly increased NADPH oxidase activity (Online Figure V). Supplementation of membrane fraction isolated from unstimulated (Control) or Ang II - stimulated BAEC with 0.5 μM mitoTEMPO did not affect NADPH oxidase activity (Online Figure V).

Effect of mitoTEMPO on $O_2^{\bullet-}$ in intact endothelial cells: In this work we have reported that supplementation of BAEC with mitoTEMPO increases $O_2^{\bullet-}$ dismutation in mitochondria (Table 1) and reduces the mitoSOX fluorescence (Figure 2, Online Figure III). To further demonstrate that mitoTEMPO scavenges mitochondrial $O_2^{\bullet-}$ we performed additional experiments in cells in which SOD2 was downregulated by siRNA.

Analysis of HAEC transfected with non-silencing siRNA (NS siRNA) showed that angiotensin II increased the $O_2^{\bullet-}$ similar to non-transfected cells (Figure 2, Online Figure VI). SOD2 depletion increased both basal and angiotensin II – stimulated mitoSOX fluorescence (Online Figure VI). MitoTEMPO significantly inhibited fluorescence of mitoSOX. These data indicate that mitoTEMPO significantly reduces mitochondrial $O_2^{\bullet-}$.

Investigation of endothelial-independent relaxation in aortic vessels: Endothelial-independent relaxation was measured by vessels relaxations to cumulative concentrations of sodium nitroprusside (SNP). Endothelium-independent relaxation was similar in vessels isolated from mice infused with saline (Sham), mitoTEMPO, angiotensin II (Ang II) or angiotensin II-infused mice treated with mitoTEMPO (Online Figure VII).

Investigation of heart rate of mice treated with angiotensin II and mitoTEMPO: The potential effect of mitoTEMPO on heart rate was evaluated using telemetry (Online Figure VIII). MitoTEMPO did not affect heart rate in normal mice, however, infusion of angiotensin II for one week increased heart rate by 10% and mitoTEMPO treatment prevented this change in the heart rate (Online Figure IVB).

References

1. Zielonka J, Hardy M, Kalyanaraman B. HPLC study of oxidation products of hydroethidine in chemical and biological systems: ramifications in superoxide measurements. *Free Radic Biol Med.* 2009;46:329-338.

2. Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK. Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radic Biol Med.* 2008;45:1340-1351.

3. Fridovich I. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J Biol Chem.* 1970;245:4053-4057.

Online Figure Legends

Online Figure I. Dose-dependent angiotensin II stimulation of endothelial $O_2^{\bullet -}$. Cellular $O_2^{\bullet -}$ production was measured in HAEC following 4 hours of treatment with angiotensin II using HPLC and DHE as described in Materials and Methods [2]. It was found that angiotensin II increased $O_2^{\bullet -}$ production in a dose-dependent manner with maximum stimulation at 200 nM concentration. Results represent mean ± SEM. **P* < 0.05 vs 0 nM, ** *P* < 0.05 vs 100 nM Ang II.

Online Figure II. Measurements of xanthine oxidase activity in the presence of mitoTEMPO (A) or mitoTEMPO-H (B). The activity of xanthine oxidase was monitored by accumulation of urate (295 nm, ε=11.000) [3] in the sample containing xanthine oxidase (20 mU/ml) and xanthine (200 μM). Data represent the mean values from three separate experiments.

Online Figure III. HPLC measurements of superoxide specific (2-OH-Mito-E^+) and non-specific (Mito-E^+) fluorescent products of mitoSOX as was previously described [1]. (A) Superoxide was generated using xanthine and xanthine oxidase and 2-OH-Mito-E^+ and Mito-E^+ were separated using HPLC. Experiments were performed without (upper chromatogram) and with (middle trace) SOD (100 U/ml). The lower panel shows the chromatogram of unreacted mitoSOX. (B) Typical HPLC chromatograms of cellular extracts obtained after incubation of HAEC with 2 μM mitoSOX for 20 minutes. (C) Bar graph showing levels of 2-OH-Mito-E^+ in unstimulated HAEC or HAEC stimulated with angiotensin II (Ang II, 4 hours, 200nM) and supplemented for 15 minutes with saline or the mitochondria-targeted SOD mimetic mitoTEMPO (25 nM). Results are mean±SEM, n=4 each. **P* < 0.05 vs control, ** *P* < 0.05 vs Ang II.

Online Figure IV. Site-specific detection of cellular and mitochondrial $O_2^{\bullet -}$ by DHE and mitoSOX using HPLC. Superoxide production was induced by NADPH oxidase activator

phorbol myristate acetate (PMA, 10 μM) or mitochondrial complex III inhibitor antimycin A (AA, 1 μM). Cellular and mitochondrial $O_2^{\bullet-}$ were measured by DHE (A) or mitoSOX (B) using HPLC as was previously described [1, 2].

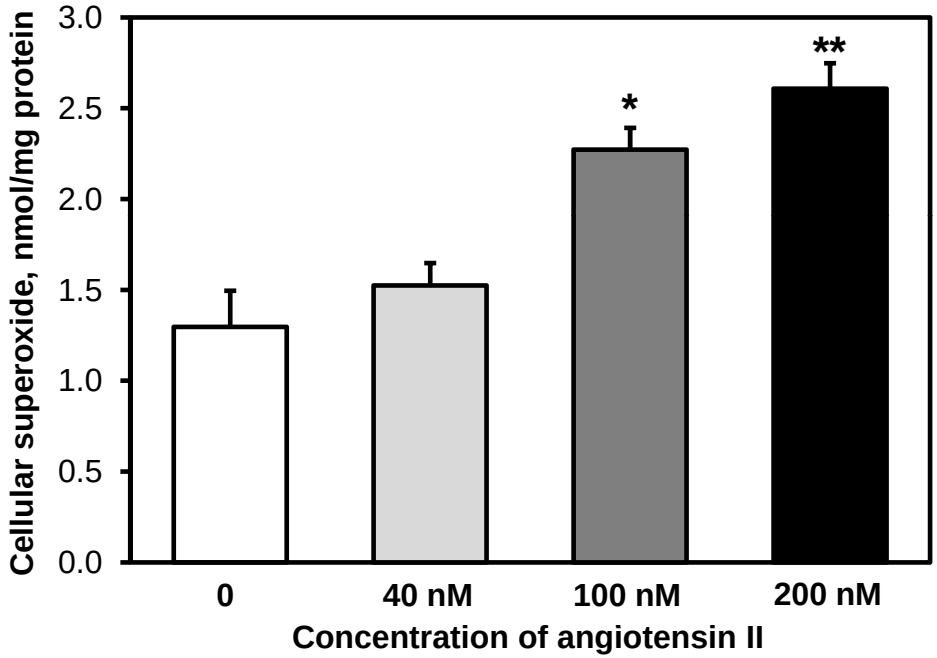
Online Figure V. NADPH oxidase activity in membrane fractions supplemented with mitoTEMPO (0.5 μM). Activity of NADPH oxidase was measured as NADPH-dependent $O_2^{\bullet-}$ production in membrane fractions using ESR as described in Materials and Methods [2]. NADPH oxidase activity was analyzed in membrane fractions of control unstimulated BAEC or BAEC stimulated with 200 nM angiotensin II for 4-hours. MitoTEMPO (0.5 μM) was applied to the membrane fractions after isolation for 30 minutes prior to measurements of NADPH oxidase activity. Direct supplementation of mitoTEMPO to membrane fractions isolated from control or angiotensin II stimulated BAEC did not affect NADPH oxidase activity. Data are average from three to six separate experiments ± SEM. *P<0.01 vs Control.

Online Figure VI. Effect of mitoTEMPO on mitochondrial $O_2^{\bullet-}$ measured with MitoSOX. HAEC were treated with siSOD2 for 72 hours and then stimulated with 200nM angiotensin II (Ang II) for 4hours. MitoTEMPO (25 nM, mT) was added for 15 minutes prior to supplemention of mitoSOX. Mitochondrial localization of MitoSOX signal was confirmed by colocalization with MitoTracker.

Online Figure VII. Effects of mitoTEMPO treatment on endothelial-independent relaxation in aortic vessels isolated from mice infused with saline (Sham), mitoTEMPO, angiotensin II (Ang II) or angiotensin II-infused mice treated with mitoTEMPO. Results represent mean ± SEM for 6-8 animals per group.

Online Figure VIII. Heart rate in C57Blk/6 mice after one week infusion with saline (Control), mitoTEMPO, angiotensin II (Ang II) or mice treated with both angiotensin II and mitoTEMPO (Ang II + mitoTEMPO. Treatment with mitoTEMPO did not affect the heart rate in the control mice. Co-infusion of mitoTEMPO and angiotensin II (0.7 mg/kg/day) attenuated angiotensin II induced increase in heart rate which was associated with antihypertensive effect of mitoTEMPO. Results represent mean ± SEM for 5 animals per group.

Online Figure I



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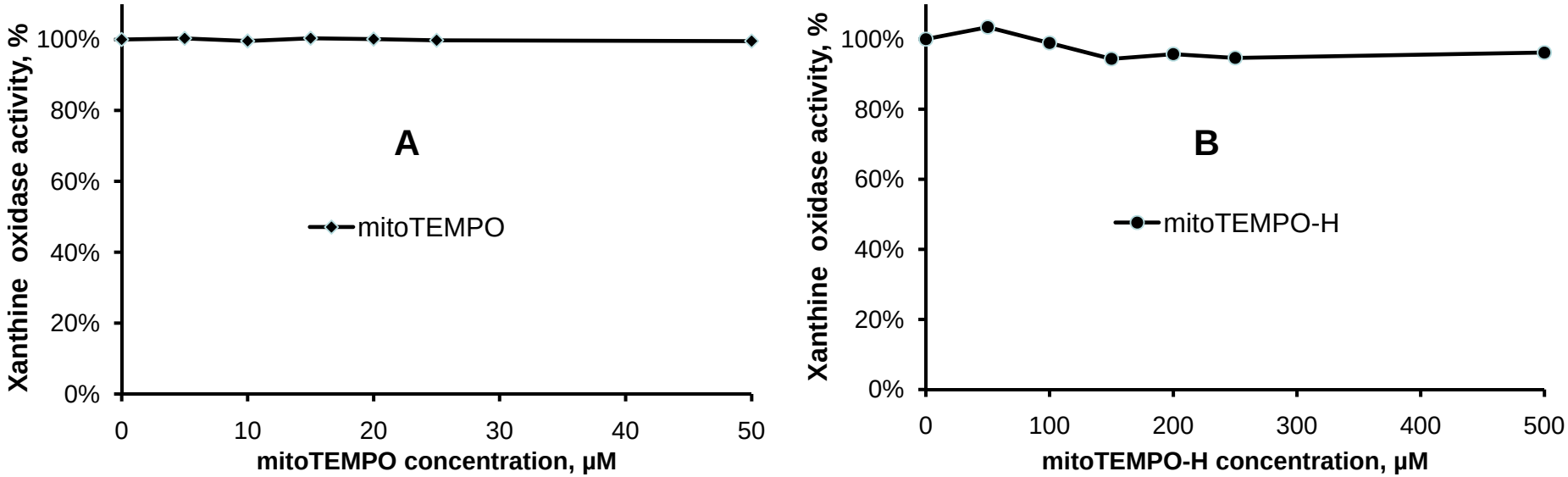

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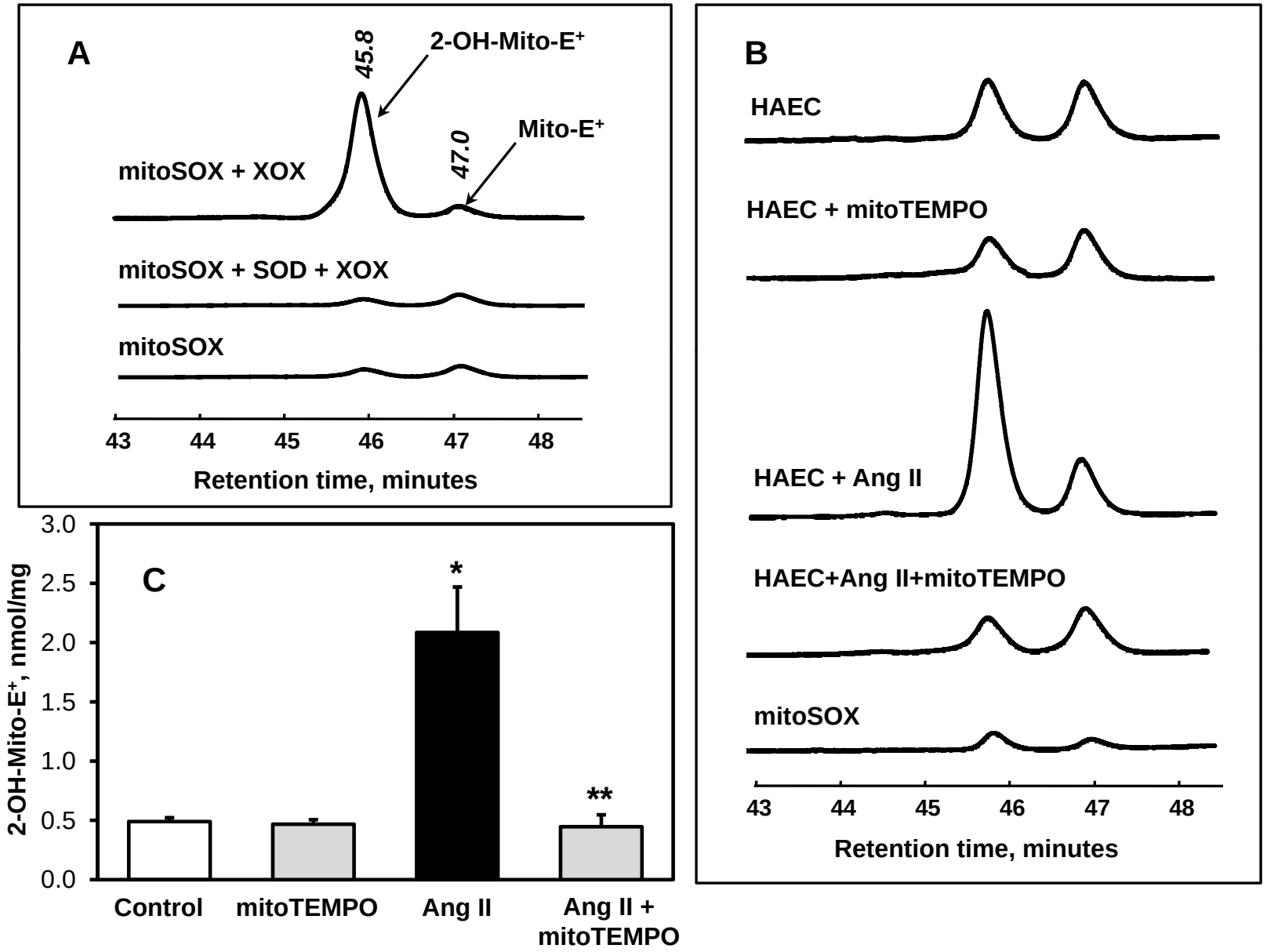

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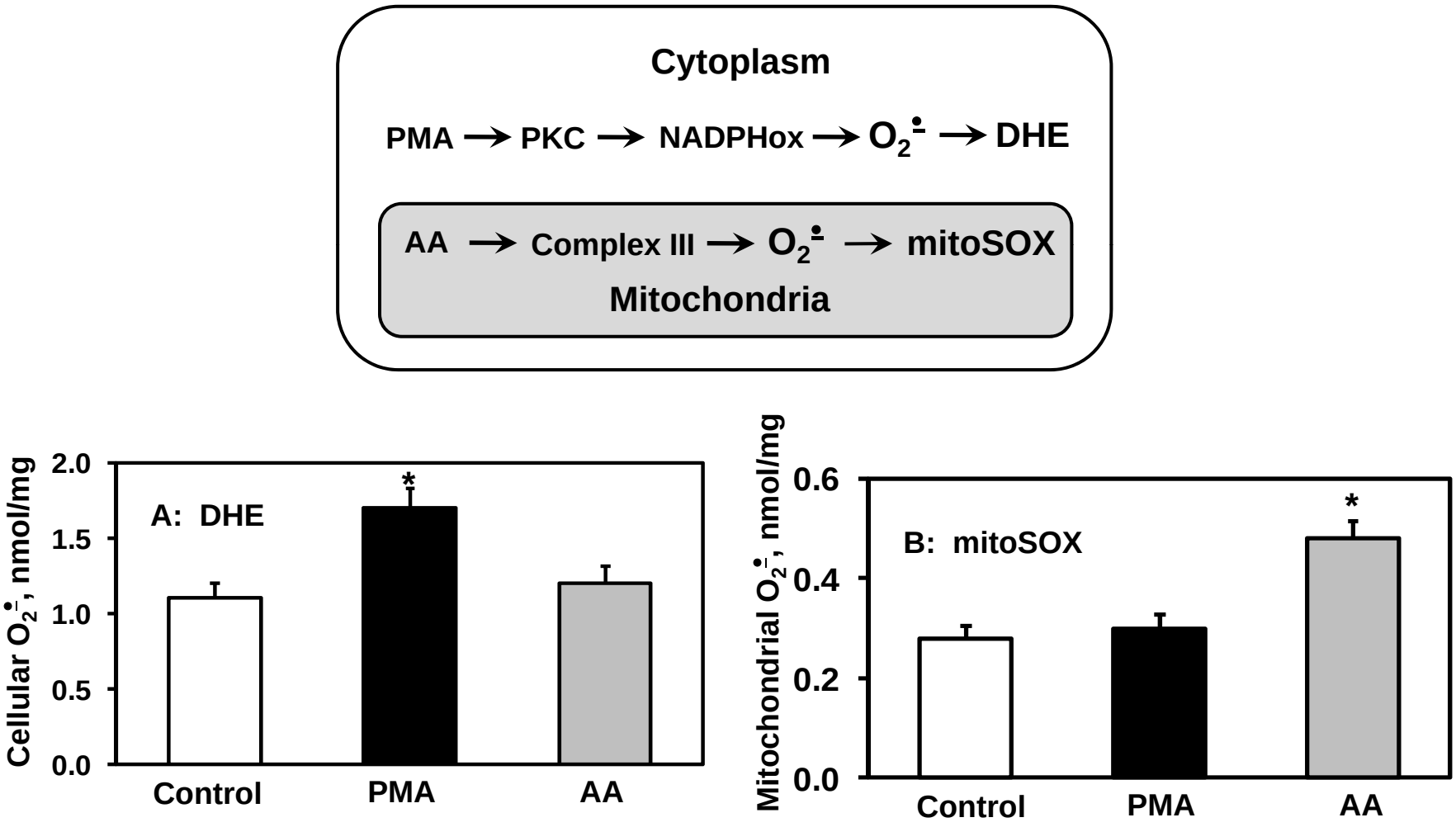

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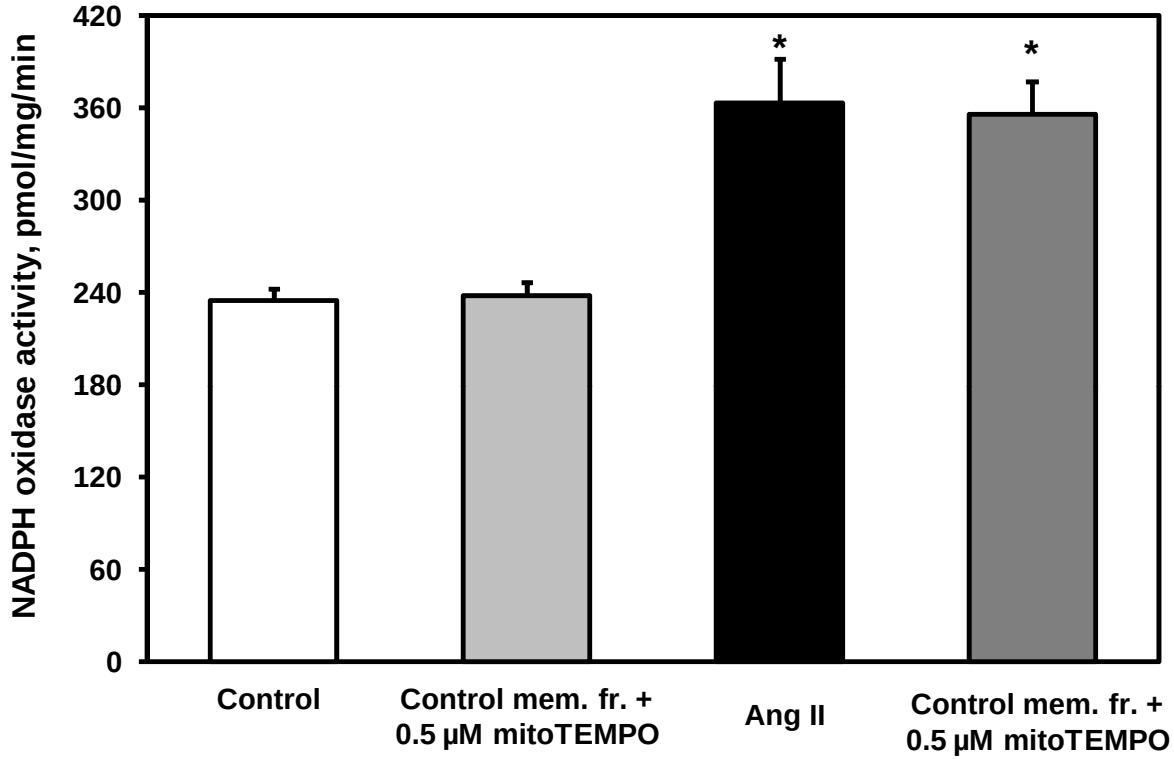

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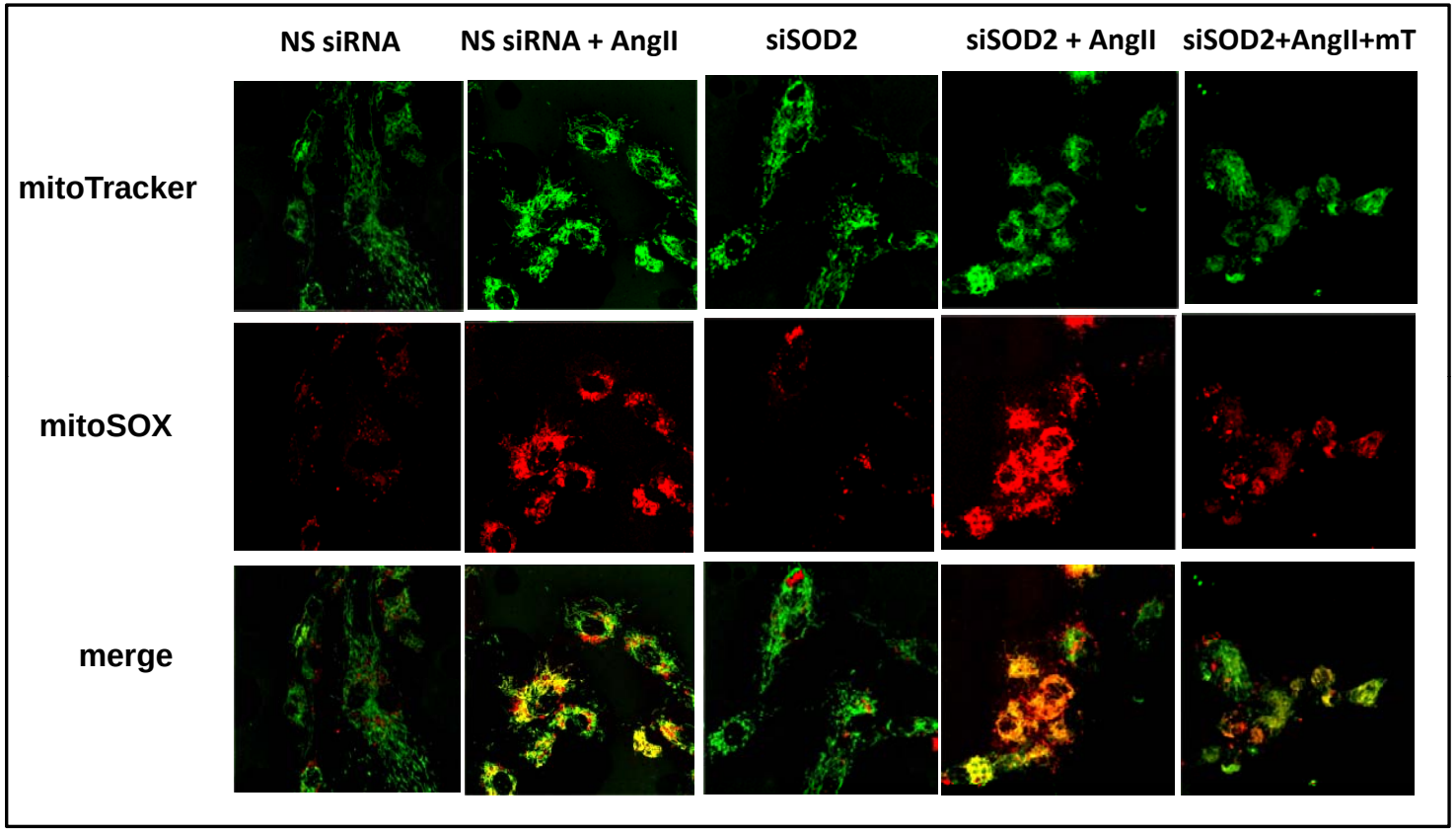

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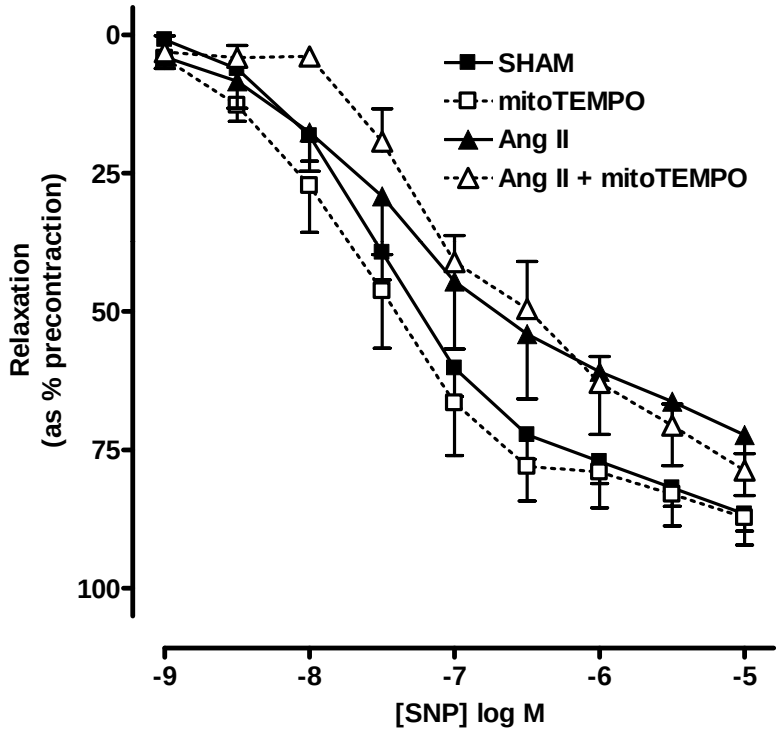

Online Figure VIII