Supplemental Methods

Neonatal mouse cardiomyocyte culture and flow cytometry

Ventricles from mouse neonates younger than 72 hours were dissected, minced, and enzymatically digested with Blendzyme 4 (45μg/ml, Roche). After enzymatic digestion, cardiomyocytes were enriched using differential pre-plating for 2 hours, then seeded on fibronectin-coated culture dishes for 24 hours in DMEM (Gibco), with 20% Fetal Bovine Serum (Sigma) and 25μM Arabinosylcytosine (Sigma). Cardiomyocytes were stimulated with Angiotensin II (1μM) for 3 hours in serum-free DMEM containing 0.5% insulin-transferrin-selenium (Sigma), 2mM glutamine, and 1mg/ml of BSA. Cardiomyocytes were simultaneously treated with either of the following: PBS control, SS-31 (1nM), N-acetyl cysteine (NAC: 0.5mM), 4-chlorodiazepam (25 μM), diazoxide (200 μM), cyclosporine(0.5 μM), or 5-hydroxydecanoate (100μM). MitoSOX (5μM) and CM-DCFCA (5μM) was incubated for 30 min at 37°C to load cardiomyocytes for measurement of mitochondrial superoxide and total cellular ROS, followed by 2 washes, with Hanks Balanced Salt Solution. Samples were analyzed by flow cytometry using excitation/emission of 488/625 nm and 488/525 nm for MitoSOX and DCFDA, respectively. Flow data was analyzed using FCS Express (De Novo Software, Los Angeles, CA), and presented as histogram distributions of MitoSOX or DCFDA fluorescence intensity.

Mouse experiments, drug delivery, echocardiography and blood pressure measurement

All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee. C57BL6 mice (J strain) were housed in a barrier specific-pathogen-free facility and maintained as described (1). Six to ten mice were included in each experimental group, including: saline, Ang, Ang + SS-31, Ang + NAC, WT, Gαq (FVBxC57BL/6 F2 hybrid, kindly provided by Dr.Gerald Dorn II) and Gαq +SS-31. A pressor dose of Ang (1.1 mg/kg/d) was continuously administered for 4 weeks using subcutaneous Alzet 1004 osmotic minipumps, with or without addition of SS-31 (3 mg/kg/d). NAC was added to drinking water (2mg/mL made fresh every week for 4 weeks) with the average dose of approximately 500mg/kg/d, as previously reported(2). Echocardiography was performed at baseline and after 4 weeks of pump implantation using a Siemens Acuson CV-70 equipped with a 13 MHz probe, as previously described(1). Under 0.5% isoflurane to reduce agitation, standard M-mode, conventional and Tissue Doppler images were taken, and functional calculations were performed according to American Society of Echocardiography guidelines. Myocardial performance index (MPI) was calculated as the ratio of the sum of isovolemic contraction and relaxation time to LV ejection time. An increase in

MPI is an indication that a greater fraction of systole is spent to cope with the pressure changes during the isovolemic phases. As a reference for SS-31 peptide effect in Ang-treated mice, we included a genetic mouse model of Rosa-26 inducible-mCAT, in which a flox-stopped mitochondrial catalase was overexpressed by tamoxifen induction of Rosa-26 promoted cre two weeks before Ang treatment.

Blood pressure was measured in a separate group of mice by telemetry using an intravascular catheter PA-C10 (DSI, MN), in which measurement was performed every three hours starting from 2 days before pump placement until 2 days after Ang pump placement, then Ang pump was removed and another new pump loaded with Ang + SS-31 was inserted 24 hours later, after the blood pressure reading returning to pre-Ang phase, followed by 2 days of recording to see if SS-31 had an effect on blood pressure.

Quantitative Pathology

Ventricular tissues were cut into transverse slices, and subsequently embedded with paraffin, sectioned, and subjected to Masson Trichrome staining. Quantitative analysis of fibrosis was performed by measuring the percentage of blue-staining

fibrotic tissue relative to the total cross-sectional area of the ventricles, by using software developed in our lab (1).

Measurement of mitochondrial protein carbonyl groups

For mitochondrial protein extraction, ventricular tissues were homogenized in mitochondrial isolation buffer (1mM EGTA, 10mM HEPES, 250mM sucrose, 10mM Tris-HCl, pH 7.4). The lysates were centrifuged for 7 minutes at 800g in 4°C. The supernatants were then centrifuged for 30 minutes at 4000g in 4°C. The crude mitochondria pellets were resuspended in small volume of mitochondrial isolation buffer, sonicated on ice to disrupt the membrane, and treated with 1% streptomycin sulfate to precipitate mitochondrial nucleic acids. The OxiSelect^{TM} Protein Carbonyl ELISA Kit (Cell Biolabs) was used to analyze 1µg of protein sample per assay. The ELISA was performed according to the instruction manual, with slight modification. Briefly, protein samples were reacted with dinitrophenylhydrazine (DNPH) and probed with anti-DNPH antibody, followed by HRP conjugated secondary antibody. The anti-DNPH antibody and HRP conjugated secondary antibody concentrations were 1:2500 and 1:4000, respectively.

Quantitative PCR

Gene expression was quantified by quantitative real time PCR using an Applied Biosystems 7900 themocycler with Taqman Gene Expression Assays on Demand, which included: PGC1-α (Mm00731216), TFAM (Mm00447485), NRF-1 (Mm00447996), NRF-2 (Mm00487471), Collagen 1a2 (Mm00483937), and ANP (Mm01255747). Expression assays were normalized to 18S RNA.

Western Immunoblots

Cardiac protein extracts were prepared by homogenization in lysis buffer containing protease and phosphatase inhibitors on ice (1.5mM KCl, 50mM Tris HCl, 0.125% Sodium deoxycholate, 0.375% Triton X 100, 0.15% NP40, 3mM EDTA). The samples were sonicated and centrifuged at 10,000g for 15 minutes in 4°C. The supernatant was collected and the concentration was determined using a BCA assay (Pierce Thermo Scientific, Rockford, IL). Total protein (25μg) was separated on NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred to 0.45 μm PVDF membrane (Millipore), and then blocked in 5% non-fat dry milk in Tris-buffer solution with 0.1% Tween-20 for 1 hour. Primary antibodies were incubated overnight, and secondary antibodies were incubated for 1 hour. The primary antibodies included: NOX-4 (Santa Cruz Biotechnology), rabbit monoclonal anti-cleaved caspase-3 (Cell Signaling), mouse monoclonal anti-GAPDH (Millipore), rabbit polyclonal phospho-p38 MAP

kinase (Cell Signaling), and mouse monoclonal anti-p38 (Santa Cruz Biotechnology). The enhanced chemiluminescence method (Thermo Scientific) was used for detection. Image Quant ver.2.0 was used to quantified the relative band density as a ratio to GAPDH (internal control). All samples were normalized to the same cardiac protein sample.

1. Dai DF, Santana LF, Vermulst M, et al. Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. Circulation 2009;119:2789-97.
2. Byrne JA, Grieve DJ, Bendall JK, et al. Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. Circ Res 2003;93:802-5.

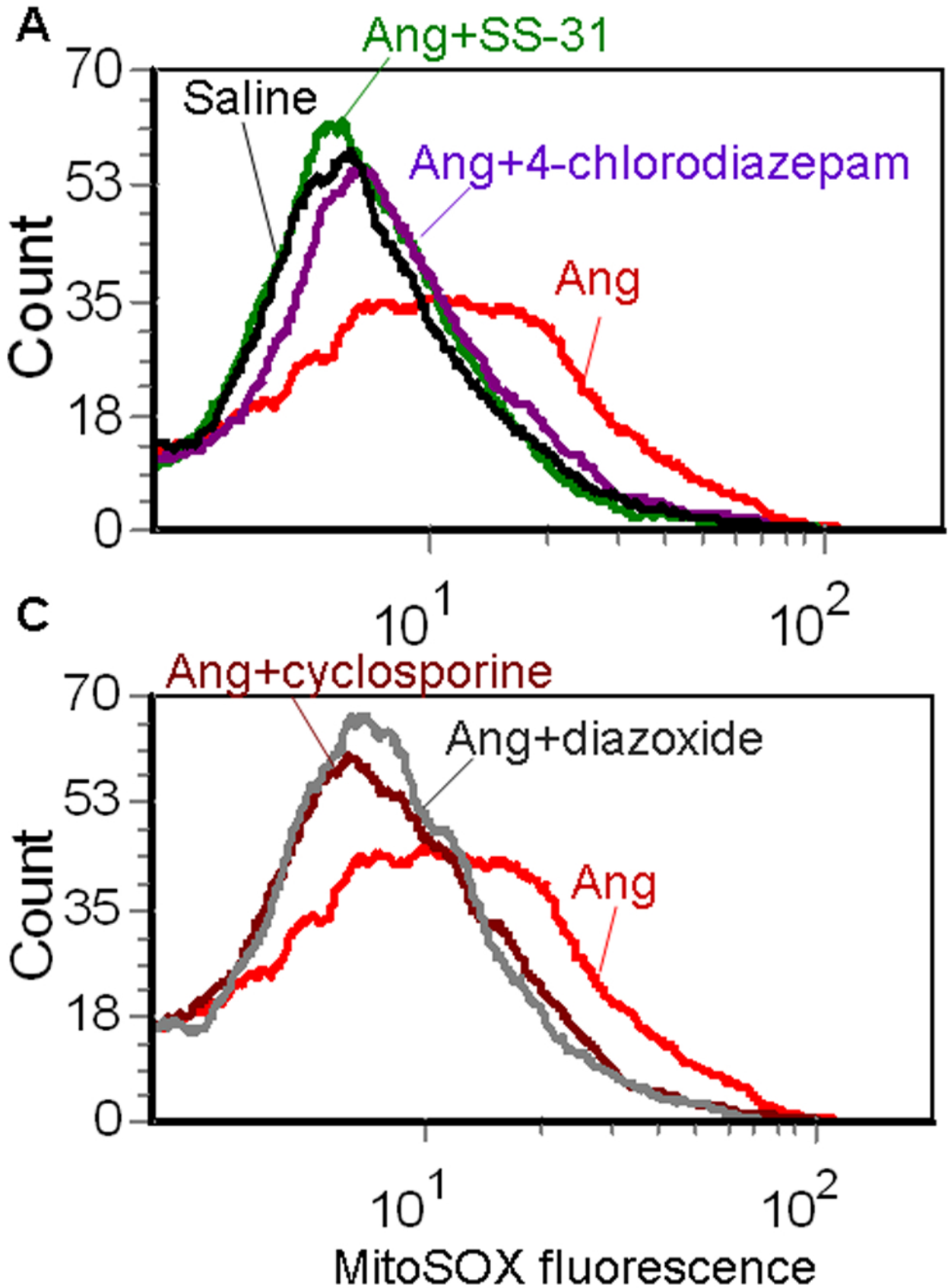
Supp fig legends:

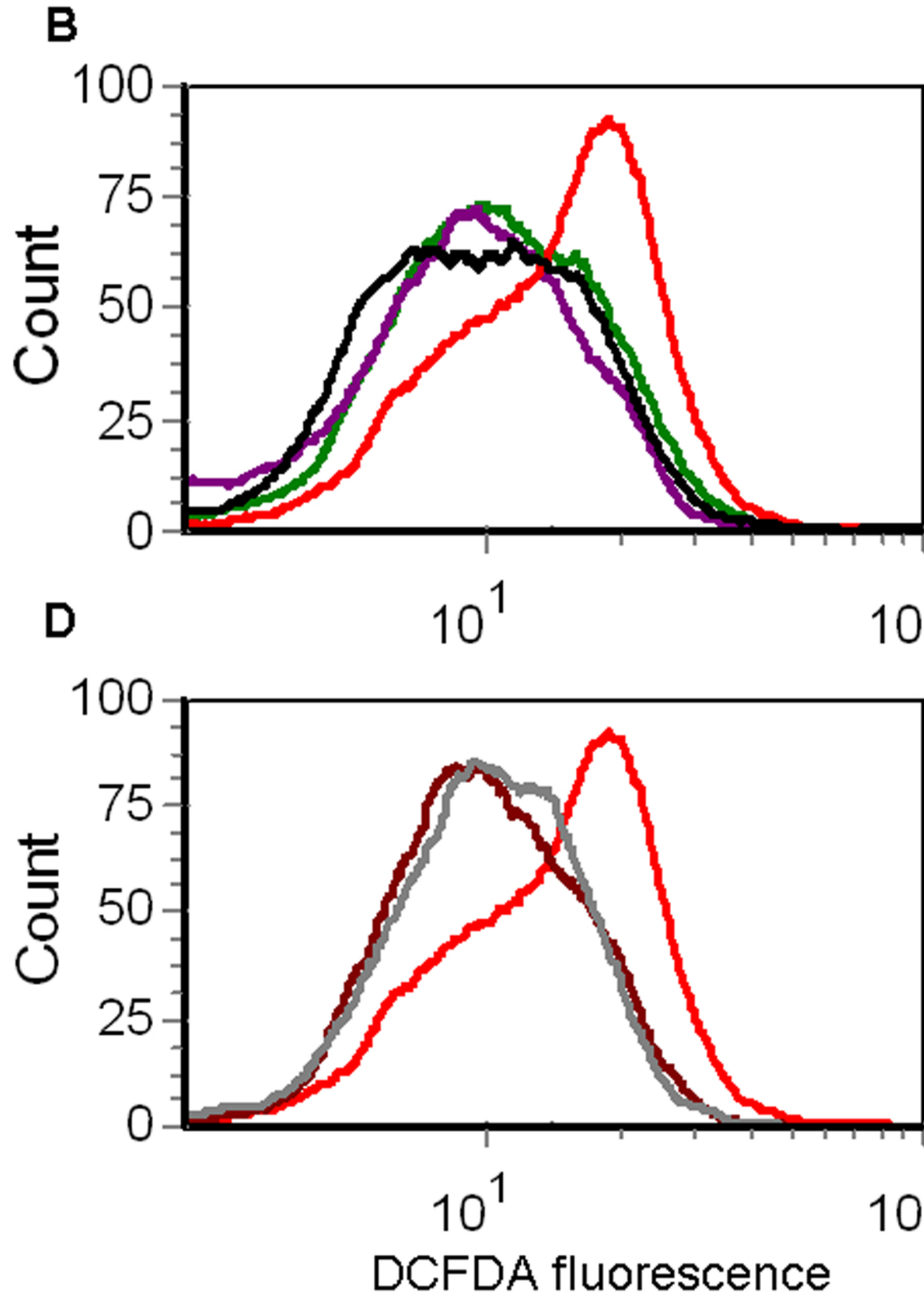
SFig1. Ang-induced MitoSOX (A,C) and DCFDA (B,D) fluorescence (red histogram) were attenuated by simultaneous treatment with 4-chlorodiazepam (top panels) or diazoxide or cyclosporine (bottom panels), similar to the treatment with SS-31, as seen by quantification of mean ± SEM of median of the fluorescence histogram (G). Treatment with mitochondrial K-ATP blocker 5HD (5-hydroxydecanoate) did not significantly affect Ang-induced ROS (E,F). n=3

SFig.2 Ang-induced NOX4 protein upregulation (A) and increase in cleaved caspase-3 (B) were not significantly attenuated by simultaneous treatment with NAC. However, NAC treatment attenuates Ang-induced phosphorylation of p38 (C), n=3-6.

SFig.3 (A, B) Both cardiac fibrosis and cardiac expression of Coll1a2 gene were not significantly altered by 4 weeks of SS-31 treatment. (C). Cardiac mitochondrial protein carbonyl content significantly increased after Ang, which was reduced by SS-31, n=5-7

SFig.1

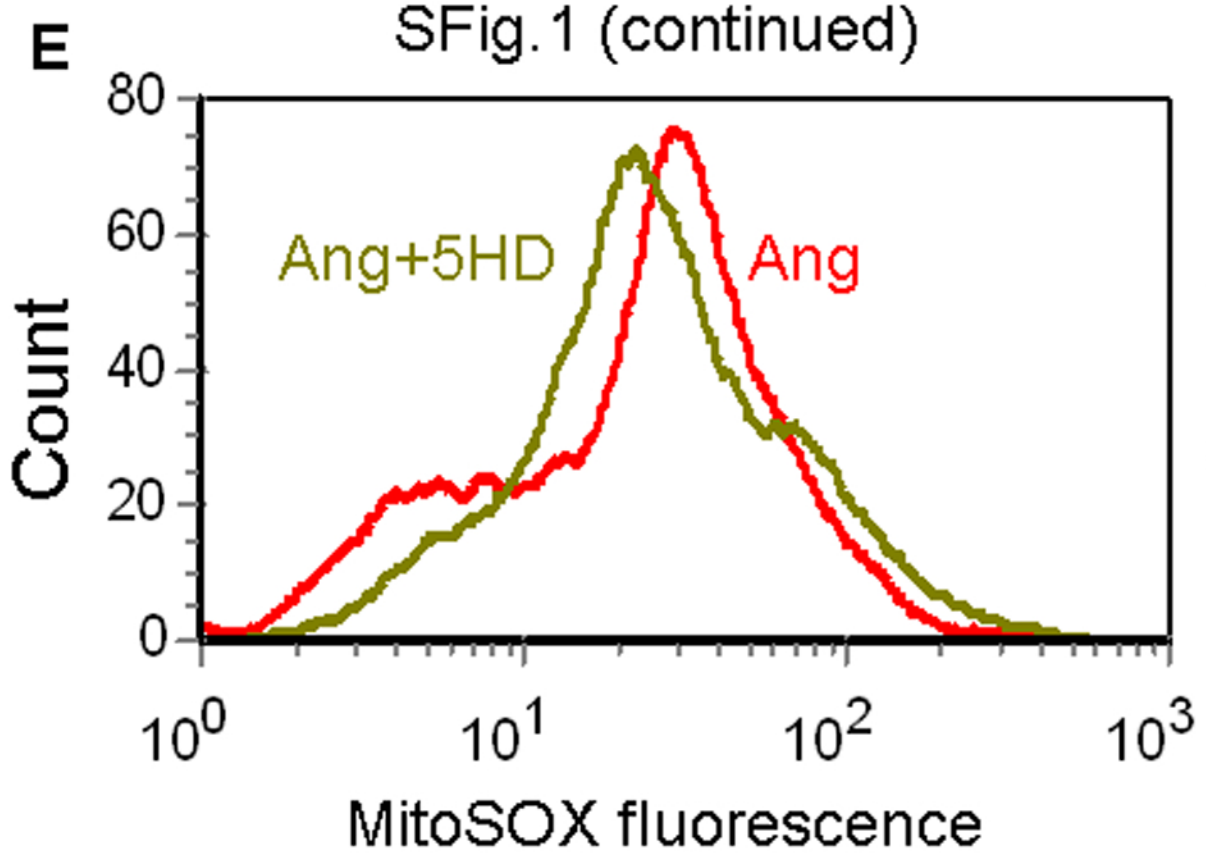

A
Ang+SS-31
Saline
Ang+4-chlorodiazepam
Ang
Count
70
53
35
18
0
10^1
10^2

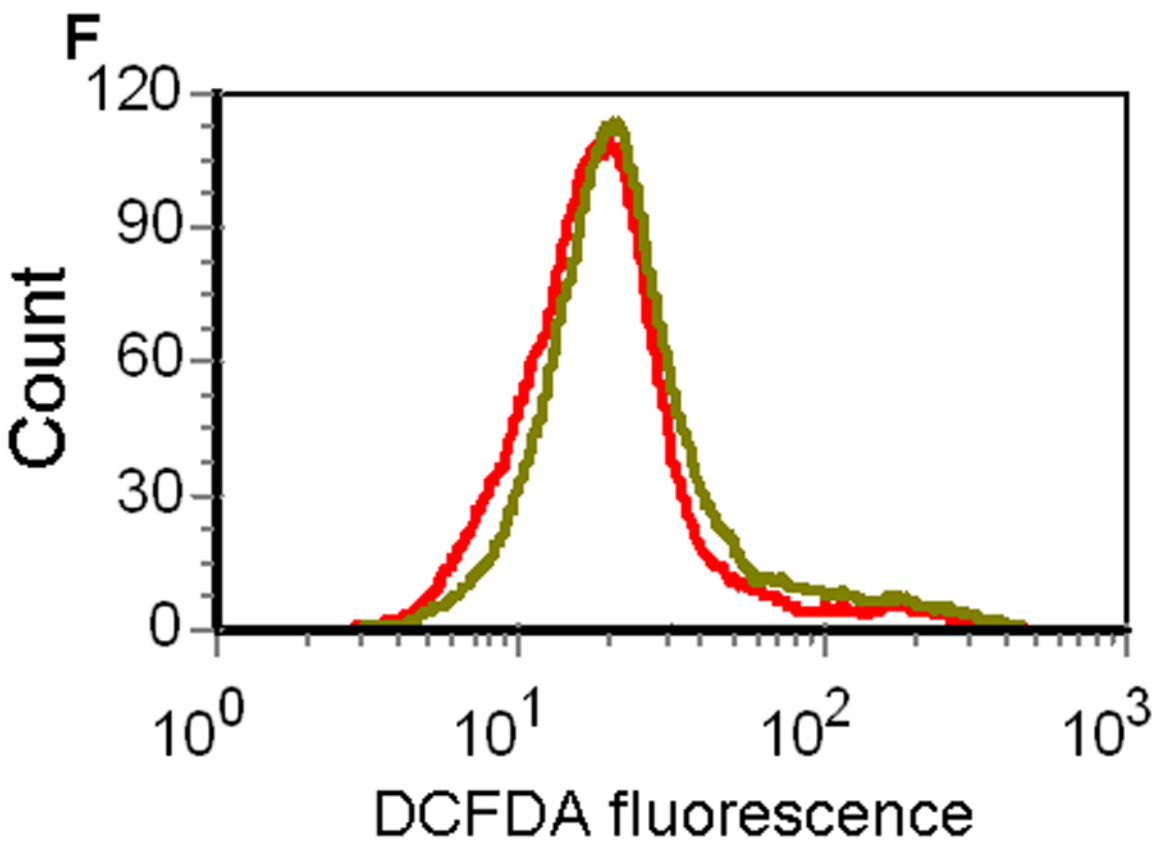

B
Count
100
75
50
25
0
10^1
10^2

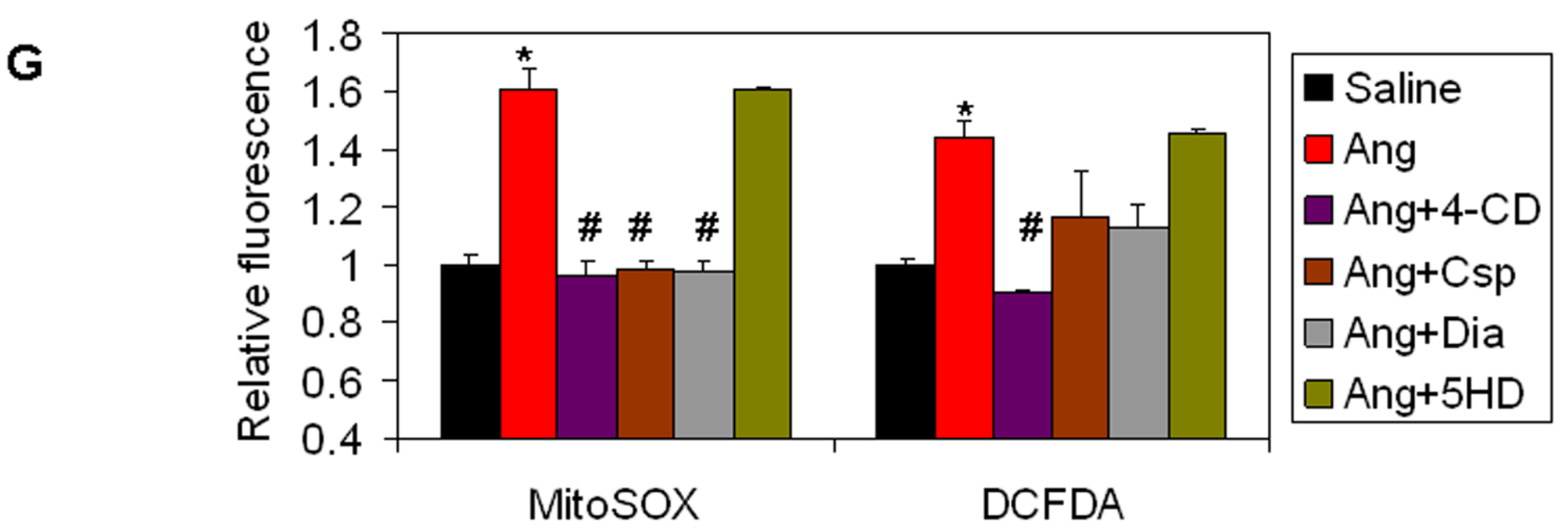
C
Ang+cyclosporine
Ang+diazoxide
Ang
Count
70
53
35
18
0
10^1
10^2
MitoSOX fluorescence

D
Count
100
75
50
25
0
10^1
10^2
DCFDA fluorescence

SFig.1 (continued)

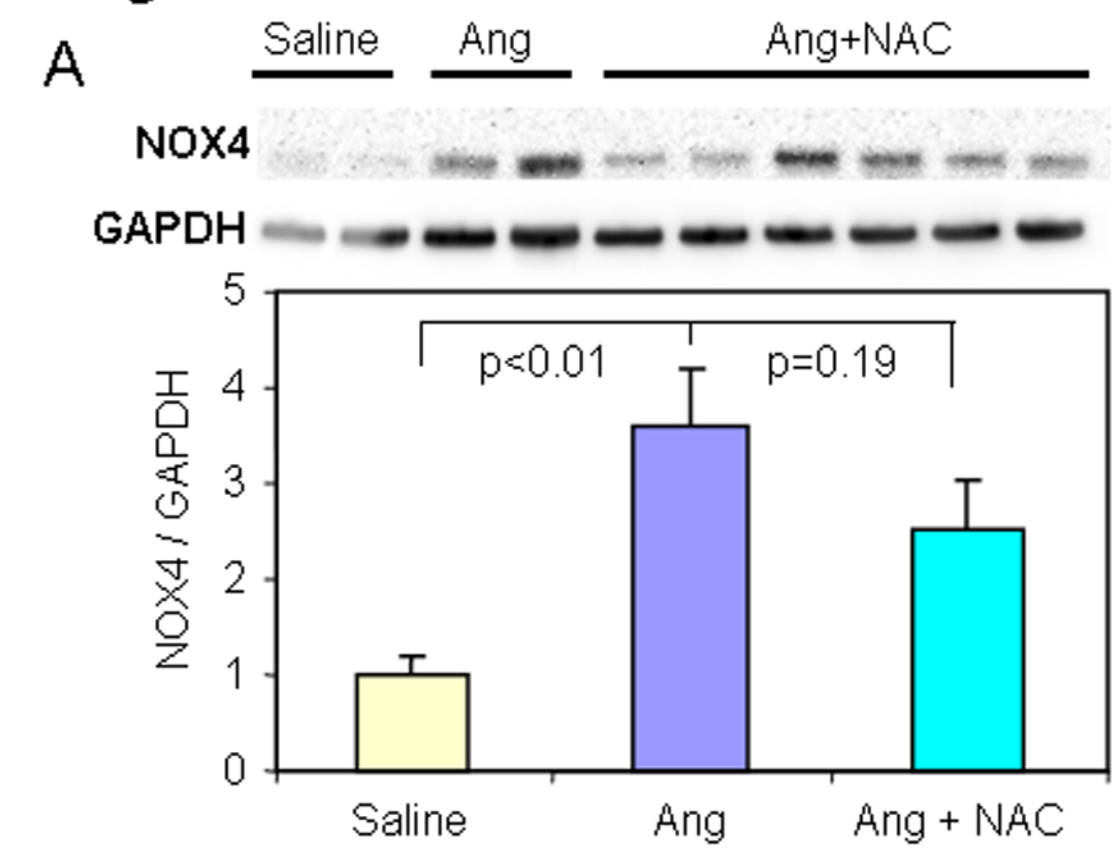


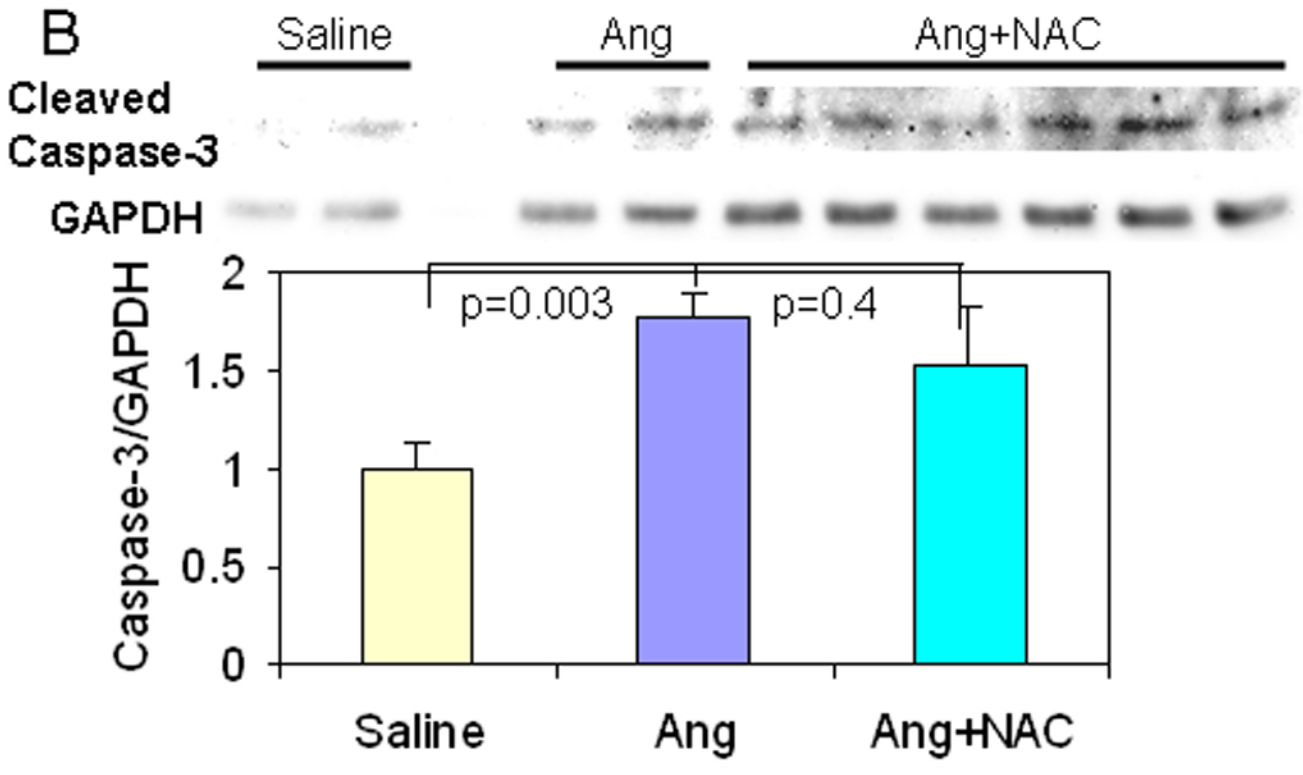


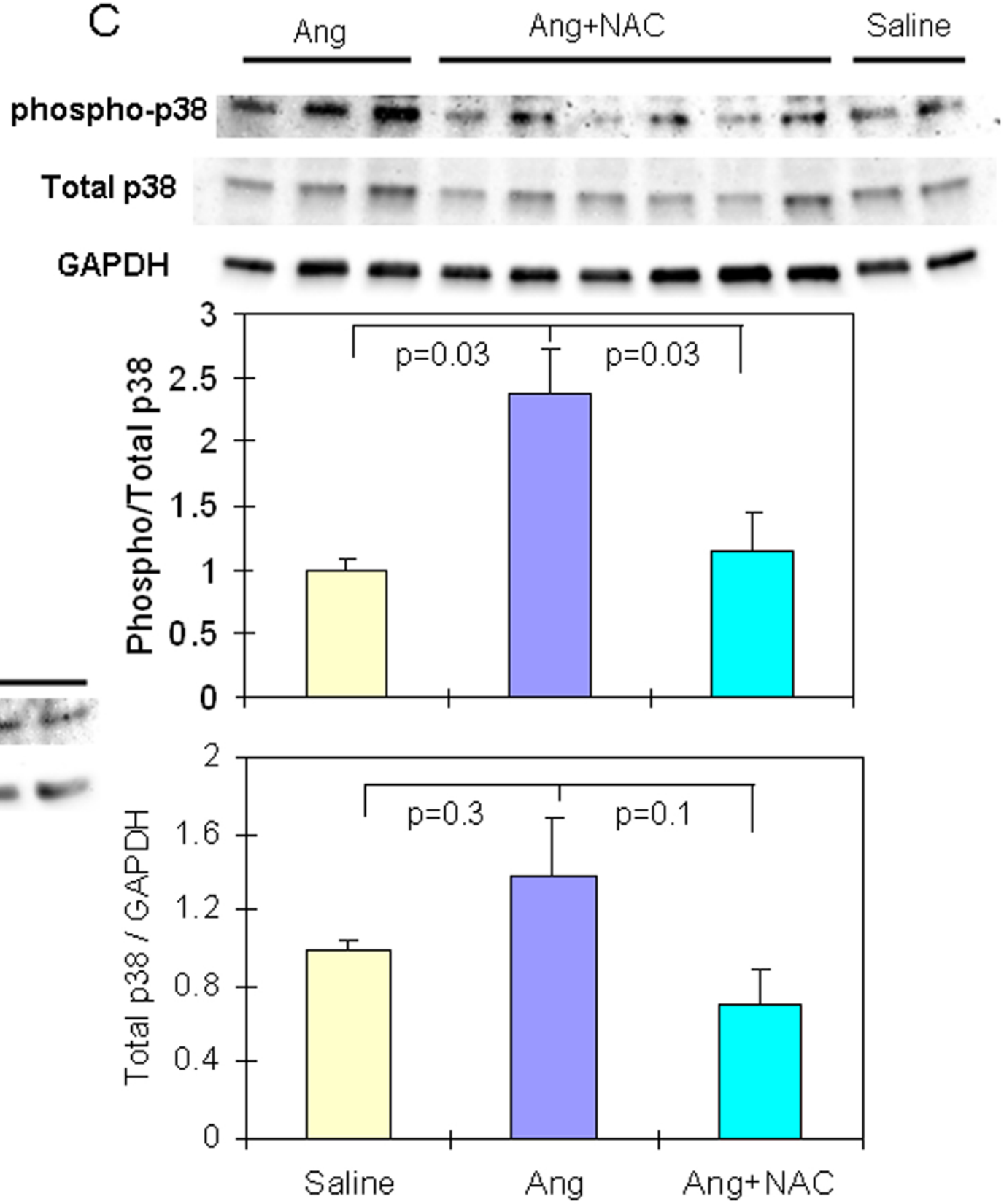


Ang-induced MitoSOX (A,C) and DCFDA (B,D) fluorescence (red histogram) were attenuated by simultaneous treatment with 4-chlorodiazepam (A,B) or diazoxide or cyclosporine (C,D), similar to the treatment with SS-31 (A,B and see Fig 1). Treatment with the mitochondrial K-ATP blocker 5HD (5-hydroxydecanoate) did not significantly affect Ang-induced ROS (E,F). Quantification of fluorescence means ± SEM is shown in panel (G), n=3. *p<0.01 vs. saline; #p<0.01 vs. Ang.

SFig.2

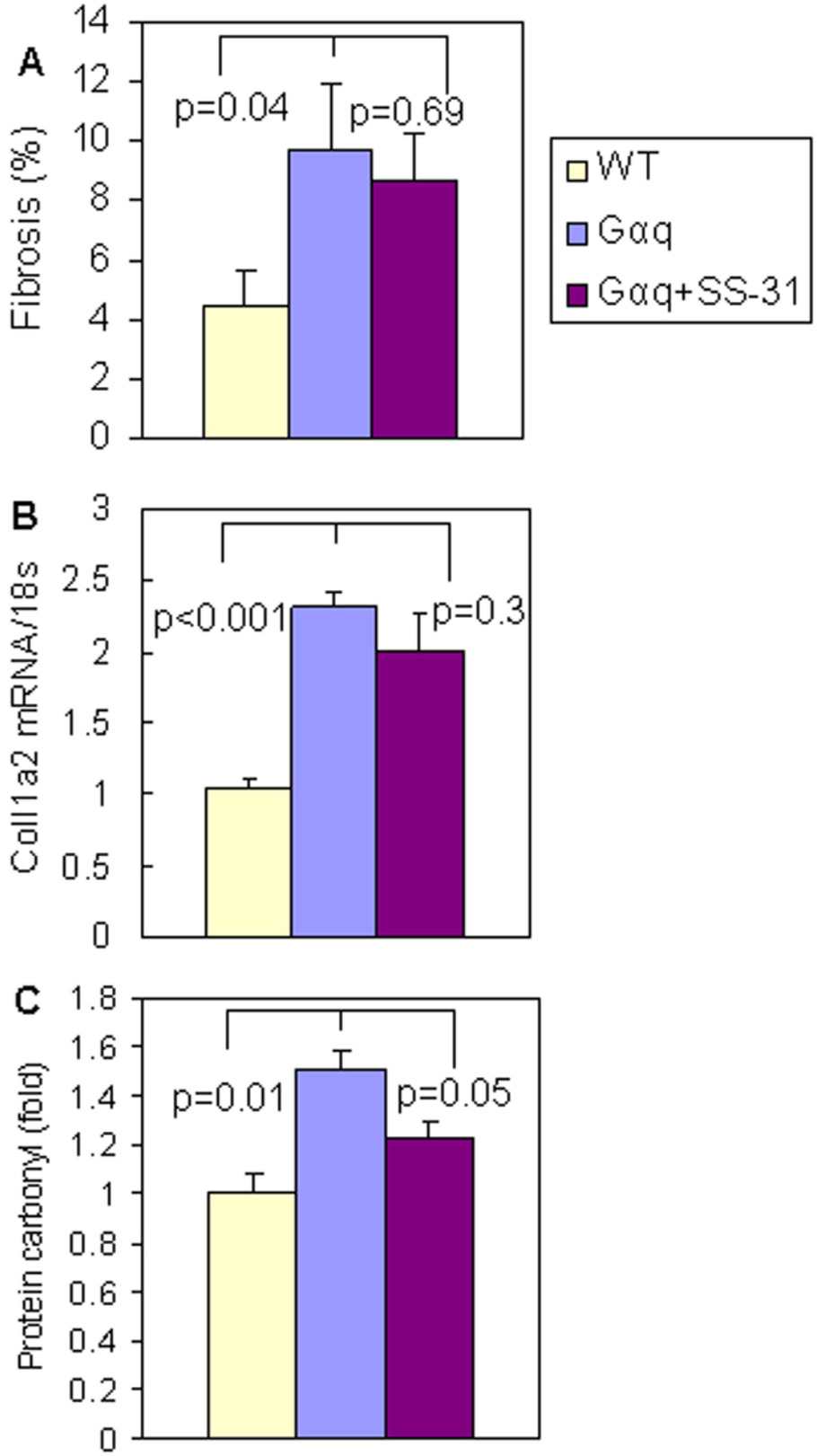






Ang-induced NOX4 protein upregulation (A) and increase in cleaved caspase-3 (B) were not significantly attenuated by simultaneous treatment with NAC. However, NAC treatment attenuated Ang-induced phosphorylation of p38 (C), n=3-6.

SFig.3



(A,B) Both cardiac fibrosis and cardiac expression of Col1a2 gene were not significantly altered by 4 weeks of SS-31 treatment. (C). Cardiac mitochondrial protein carbonyl content significantly increased after Ang II, which was reduced by SS-31, n=5-7.