Impact of 17β-estradiol on complex I kinetics and H2O2 production in liver and skeletal muscle mitochondria

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Naturally or surgically induced postmenopausal women are widely prescribed estrogen therapies to alleviate symptoms associated with estrogen loss and to lower the subsequent risk of developing metabolic diseases, including diabetes and nonalcoholic fatty liver disease. However, the molecular mechanisms by which estrogens modulate metabolism across tissues remain undefined. We have previously reported that 17β-estradiol (E2) exerts antiadipogenic effects in ovariectomized (OVX) mice by protecting mitochondrial and cellular redox function in skeletal muscle. The liver is another key tissue for glucose homeostasis and a target of E2 therapy. Thus, in the present study we determined the effects of acute loss of ovarian E2 and E2 administration on liver mitochondria. In contrast to skeletal muscle mitochondria, E2 depletion via OVX did not alter liver mitochondrial respiratory function or complex I (CI) specific activities (NADH oxidation, quinone reduction, and H2O2 production). Surprisingly, in vivo E2 replacement therapy and in vitro E2 exposure induced tissue-specific effects on both CI activity and on the rate and topology of CI H2O2 production. Overall, E2 therapy protected and restored the OVX-induced reduction in CI activity in skeletal muscle, whereas in liver mitochondria E2 increased CI H2O2 production and decreased ADP-stimulated respiratory capacity. These results offer novel insights into the tissue-specific effects of E2 on mitochondrial function.

With menopause occurring at age ~50 and a life expectancy in the United States of ~80 years (1), women spend the latter third of their lives in a low 17β-estradiol (E2)3 state, which is associated with an elevated risk of developing obesity, type 2 diabetes, and cardiovascular disease (2). Estrogen-based hormone therapies (ET, estrogens ± progestins) have been recommended since the 1950s to help ameliorate menopausal symptoms and reduce the risk of developing chronic diseases (3). Although the use of ET curtailed in the early 2000s after the Women's Health Initiative Trial reported that ET increased the risks of oncogenicity and cardiovascular events (4), subsequent studies and meta-analyses later confirmed that ET does indeed reduce all-cause mortality (5). Today, the North American Menopause Society endorses ET based on an algorithm-derived benefit/risk ratio (6), especially for those <60 years of age and/or within <10 years postmenopause (7). However, the molecular mechanism(s) by which ET affects metabolism across tissues remains poorly understood. In addition, various formulations of custom-compounded “bioidentical” hormone therapies are used by 1–2.5 million women per year in the United States alone (8), despite a lack of evidence-based support for their safety and disease prevention claims (9) or U.S. Food and Drug Administration approval (10).

In a wide range of nonreproductive tissues, E2 has consistently been shown to modulate several parameters of mitochondrial function, including oxidative phosphorylation (11), ATP production, membrane potential (12, 13), Ca2+ homeostasis (14), and mitochondrial morphology dynamics (15, 16). In skeletal muscle, E2 localizes to the inner mitochondrial membrane, where it lowers membrane viscosity, which, in turn, promotes bioenergetic function, cellular redox balance, and insulin sensitivity (17). Many of the beneficial effects of E2 appear to be mediated through improved complex I (CI) activity, which is curios given that exposure of liver mitochondria to E2 reportedly inhibits CI activity (18). Combined, these findings raise the possibility that the effects of E2 on mitochondrial function may be tissue-specific (19). Similar to muscle, E2 given in vivo localizes to mitochondria in the liver (20), although the functional impact is unknown. Decreased circulating E2, as well as aging, is associated with declining liver function and increased risk of liver disease (21), particularly the development of nonalcoholic fatty liver disease (NAFLD) (22, 23). Although E2 administration in OVX rodents decreases adiposity (24) and reverses the development of fatty liver (25), postmenopausal women diagnosed with NAFLD do not show improvements with ET (26). In fact, ET is normally contraindicated in women with active liver disease or a history of liver disease (7, 27). Taken together, these data suggest that the effects of E2 may not only be tissue-specific but also dependent on the pathophysiology of the tissue.

In the present study, ex vivo and in vitro experiments were performed to assess and compare the targeted effects of E2 loss and E2 replacement on the kinetics and H2O2-emitting potential of CI in mitochondria from both liver and skeletal muscle. Overall, the findings suggest that E2 treatment is beneficial in restoring CI kinetics in skeletal muscle after ovarian E2 depletion.
In contrast, liver mitochondrial function is minimally impacted by E2 depletion, whereas E2 treatment unexpectedly increases CI-mediated H$_2$O$_2$ production and decreases OXPHOS capacity.

**Results**

**Hepatic cellular redox balance is not affected 4 weeks after OVX ± ET**

Young, sexually mature (10-week-old) female mice were studied 2 weeks after OVX (OVX-2w) and after an additional 2 weeks ± E2 treatment (OVX-4w(E2) and OVX-4w(ctl), respectively). Loss of circulating ovarian E2 was confirmed by reduced uterine weights relative to normally cycling females in the proestrus stage (NC-Pro) of the estrus cycle as previously described (17). In contrast with skeletal muscle (17), 2–4-week OVX did not affect cellular redox balance in the liver as evidenced by the lack of change in redox state of peroxiredoxins (Fig. 1, A and B) and the ratio of GSH/GSSG (Fig. 1, C–E). GSH reductase activity was increased in OVX mice treated with E2 (Fig. 1F), consistent with an E2-induced up-regulation of antioxidant enzymes in the liver (29).

**ET, but not OVX, affects mitochondrial CI and CIII function in liver**

OVX ± E2 therapy did not alter mitochondrial content in liver, as demonstrated by unchanged citrate synthase activity...
and expression levels of the respiratory complexes (Fig. 2, A and B). We recently found that loss of E2 from OVX decreases mitochondrial CI, CIII, CI+/H11001 III, and CII+/H11001 III activity in skeletal muscle (17). In sharp contrast, the specific activities of CI, CIII, and CIV, as well as electron transfer between CI and CIII and between CII and CIII, were not affected in liver mitochondria after ovarian E2 withdrawal (Fig. 2C). Also in contrast to muscle (17), CII specific activity was decreased by ~30% in liver mitochondria after OVX and was not rescued by E2. Although E2 treatment increased the individual specific activities of CI and CIII by ~40% (p < 0.05) and ~48% (p < 0.005), respectively, the accompanying CI to CIII activity remained the same.

Tissue-specific effects of OVX and ET on CI activity

To further evaluate the potential tissue-specific effects of E2 on CI kinetics and \( \text{H}_2\text{O}_2 \) production, the steady-state rates of NADH oxidation, decylubiquinone (DCU, a soluble substrate analog of coenzyme Q) reduction and \( \text{H}_2\text{O}_2 \) production were measured in fractured liver and skeletal muscle mitochondria from NC-Pro and OVX mice (Fig. 3). The NADH (I\(_c\)) and quinone (I\(_o\)) binding sites are separated by a ~90 Å tunnel containing eight redox-sensitive iron–sulfur clusters that connect the primary electron acceptor FMN to the quinone-binding site (30, 31). Overall, the maximal rate of CI-mediated NADH oxidation in NC-Pro control mice was ~2-fold higher in liver compared with skeletal muscle mitochondria (Fig. 3, A versus D), whereas the maximal rate of quinone reduction was comparable between tissues (Fig. 3, B and E). Maximal rates of \( \text{H}_2\text{O}_2 \) production by CI were also higher in liver versus muscle mitochondria (Fig. 3, C versus F).

CI activity responded differently to ovarian E2 depletion and E2 therapy in liver and skeletal muscle mitochondria. In liver, E2 depletion via OVX did not affect either the maximal rate of CI NADH oxidation or quinone reduction (Fig. 3, A–C). However, E2 treatment increased the maximal rate of NADH oxidation in OVX compared with NC-Pro control mice (Fig. 3A), whereas the maximal rate of quinone reduction remained unchanged (Fig. 3B). In skeletal muscle, the maximal rate of NADH oxidation was reduced in mitochondria isolated from OVX mice but was restored to NC-Pro levels with E2 treatment (Fig. 3D). In contrast, the maximal rate of quinone reduction remained unchanged after OVX but was significantly increased with E2 treatment (Fig. 3E). Finally, in liver mitochondria CI \( \text{H}_2\text{O}_2 \) production was not affected after either 2- or 4-week OVX but was surprisingly increased by 2-fold after E2 treatment (Fig. 3F). Consistent with prior findings (17), \( \text{H}_2\text{O}_2 \) production in mitochondria isolated from skeletal muscle was elevated in OVX mice relative to NC-Pro but restored to NC-Pro levels by E2 treatment (Fig. 3F).

Tissue-specific effects of OVX and in vitro E2 treatment on CI sites

I\(_c\) and I\(_o\) are the two major sites of superoxide/\( \text{H}_2\text{O}_2 \) production in CI (32) (Fig. 4A). In light of the tissue-specific response of CI activity, we next evaluated the influence of E2 on superoxide/\( \text{H}_2\text{O}_2 \) production at sites I\(_c\) and I\(_o\) of CI in each tissue. Freshly isolated liver and skeletal muscle mitochondria from NC-Pro and OVX-2w mice were exposed to a low (1 mM) or high (30 mM) concentration of E2 \textit{in vitro}, and \( \text{H}_2\text{O}_2 \) production was determined at each of the respective CI sites. Inhibitors to the
mitochondrial antioxidant enzymes thioredoxin reductase and GSH reductase were included to permit direct detection of $\text{H}_2\text{O}_2$ production (33). The maximal $\text{H}_2\text{O}_2$ production at site IF was measured using malate as substrate in the presence of rotenone (32). ATP and aspartate were included to prevent $\text{H}_2\text{O}_2$ generation from 2-oxoglutarate dehydrogenase, a potential site of electron leak when malate is the only substrate (32).

For the IQ site, we determined the maximal succinate-supported $\text{H}_2\text{O}_2$ production sensitive to $\text{N}$-cyclohexyl-4-(4-nitrophenoxy)-benzenesulfonamide, which inhibits electron leak at IQ without affecting oxidative phosphorylation (34). Consistent with a previous report (35), overall maximal $\text{H}_2\text{O}_2$ production was an order of magnitude lower at site IF than IQ in both liver and skeletal muscle mitochondria from control NC-Pro mice (Fig. 4). Interestingly, despite differences in the catalytic rates of NADH oxidation and DCU reduction at sites I$_F$ and I$_O$ respectively (Fig. 3), maximal $\text{H}_2\text{O}_2$ production was similar between the two tissues (Fig. 4, B versus D and C versus E). In liver mitochondria, OVX did not affect maximal $\text{H}_2\text{O}_2$ production at either site I$_F$ or I$_O$ (Fig. 4, B and C). Preincubation (15 min) of liver mitochondria from OVX mice with either low or high concentrations of E2 tended to increase maximal $\text{H}_2\text{O}_2$ production at site I$_O$ only (Fig. 4C). By contrast, in mitochondria from skeletal muscle, maximal $\text{H}_2\text{O}_2$ production at site I$_F$ was ~2-fold higher in OVX relative to NC-Pro mice ($p < 0.05$) but was restored by preincubation with E2 (Fig. 4D). Similarly, maximal $\text{H}_2\text{O}_2$ production at site I$_O$ was slightly elevated after OVX and restored by preincubation with E2 (Fig. 4E). Overall, these results suggest that loss of ovarian E2 in vivo and E2 replacement in vitro (at nanomolar concentrations as in Ref. 17) directly impacts CI-derived maximal $\text{H}_2\text{O}_2$ production and topology in a tissue-specific manner.

Effects of in vitro E2 exposure on CI-supported respiration

To test whether in vitro treatment with E2 affects mitochondrial respiration supported by CI-linked substrates (glutamate/malate), oxygen consumption rates ($\text{O}_2$) were determined in liver and muscle mitochondria from NC-Pro and OVX-2w mice exposed (15 min) to increasing concentrations of E2. In control mice prior to addition of E2, maximal ADP-stimulated CI-supported $\text{O}_2$ was ~14-fold lower in liver versus skeletal muscle mitochondria (Fig. 5, A and B). Addition of the CII-linked substrate succinate to liver mitochondria generated a higher maximal ADP-stimulated $\text{O}_2$ response, but this was still well below the respiratory capacity of skeletal muscle mitochondria. OVX-induced loss of E2 decreased maximal CI-supported $\text{O}_2$ in skeletal muscle, but not liver mitochondria. We previously found that acute in vitro incubation of skeletal muscle mitochondria from OVX mice with nanomolar concentrations of E2 replenishes membrane E2 content but fails to restore maximal $\text{O}_2$ (17). In the present study, acute in vitro exposure to low nanomolar concentrations of E2 did not increase CI-supported $\text{O}_2$ in skeletal muscle mitochondria from OVX mice (Fig. 5B), consistent with the notion that restoration of mitochondrial respiratory capacity by in vivo E2 therapy may involve genomic mechanisms. Interestingly in
liver, exposure to low nanomolar concentrations of E2 induced an inhibitory effect on maximal ADP-stimulated $J_O^2$ (Fig. 5A). Incubation with higher, supraphysiological E2 concentrations ($30 \mu M$) inhibited CI-linked $J_O^2$ by 35–50% in mitochondria from both liver and skeletal muscle of NC-Pro and OVX mice, possibly reflecting inhibition of the FMN cofactor in CI (18), $F_oF_1$-ATPase (12), and/or biophysical disruption of the membrane.

**Discussion**

Loss of circulating ovarian E2 results in diverse physiological and biochemical responses across the body (36). *In vitro*, E2...
Estrogen and complex I in liver and skeletal muscle

Figure 5. Effects of acute in vitro E2 exposure on CI-supported respiration in liver and SM mitochondria. JO2 measured in isolated mitochondria from liver (A) and SM (B) mitochondria from NC-Pro or OVX-2w mice preincubated for 15 min ± increasing concentrations of E2. The substrates added in sequence were 10 mM glutamate + 2 mM malate, 1 mM ADP and for liver 10 mM succinate, 10 μM rotenone. The data are means ± S.E. *, p < 0.05; **, p < 0.005 versus NC-Pro from independent t tests. Comparisons between increasing E2 concentrations within each group were performed using paired t tests versus 0 E2.1, p < 0.05; !!, p < 0.005 (n = 4 mice/group).

elicits different effects on mitochondria depending on the tissue from which the mitochondria are isolated (19), suggesting that the influence of E2 on mitochondrial function may at least partially account for its diverse effects across tissues in vivo. To further explore this possibility, the present study investigated the effects of short-term ovarian E2 depletion and E2 therapy on mitochondrial redox function and respiratory complex activities in both liver and skeletal muscle. In contrast to the loss of function evident in mitochondria from skeletal muscle with E2 depletion (17), mitochondria from liver appear to be relatively resilient to the loss of E2, as evidenced by the lack of significant change in mitochondrial respiratory capacity or cellular redox state. A notable exception was a decrease (~30%) in CI specific activity that persisted even after administration of E2. However, both liver and skeletal muscle mitochondria responded to E2 treatment with marked, albeit different, effects on CI activity and H2O2 production. A summary of the effects of E2 loss and E2 replacement on indices of mitochondrial function from both tissues is shown in Table 1.

The mechanism(s) behind tissue specificity of E2 action on mitochondrial function remains unknown. Our previous findings linked a direct effect of E2 on mitochondrial membrane viscosity to improved CI function in skeletal muscle (17). The extent to which a similar mechanism of action is present in other tissues is unclear, although E2-mediated increases in mitochondrial CI-supported O2 have been previously reported in other tissues with high CI-linked mitochondrial respiratory capacity, including brain (11) and cardiac muscle (37, 38). The impact of E2 loss and replacement on liver mitochondrial function, however, appears to be comparatively modest and may simply reflect tissue-specific heterogeneity in E2 content and/or varying dependence on membrane-bound estrogen receptor signaling (39, 40).

The potential effect of exogenously administered E2 on the liver also depends on the type of estrogen and the dose and route of administration (41, 42). In the present study, E2 was administered transdermally via a subcutaneous mini osmotic pump that, compared with oral administration, limits exposure of the liver and allows for a more physiological delivery of E2 to nonhepatic tissues (42). In fact, oral administration of conjugated estrogens has been associated with higher hepatic toxicity because of increased hepatic synthesis of triglycerides and coagulation and inflammatory factors (43). Inherent differences in mitochondrial lipid and/or protein composition (44–46), which influence the biophysical properties of membranes, may have also contributed to the different responses to E2 depletion and replacement between the two tissues.

The I1 site in CI is a significant contributor to H2O2 production during forward electron flow (32). We previously found that OVX induces an increase in the mitochondrial H2O2 emitting potential and an oxidative shift in the cellular redox environment in skeletal muscle, accompanied by a decrease in CI function (17). In the present study, JH2O2 production was increased specifically at site I1 after OVX, suggesting that loss of E2 increases the susceptibility of site I1 to leak electrons in skeletal muscle mitochondria. Although the mechanism is unclear, either an increase in the sensitivity of site I1 to the NADH/NAD+ ratio and/or a “backup” in downstream electron flow increasing the reducing pressure at site I1 could explain this effect (47, 48). Regardless of the mechanism, E2 treatment in vitro reversed the increased susceptibility of site I1 to leak electrons and in vivo enhanced both CI NADH oxidation and quinone reduction rates, lowering overall JH2O2 production. These effects of E2 loss and treatment were unique to skeletal muscle mitochondria and are consistent with prior findings, suggesting that the decrease in membrane microviscosity induced by the presence of E2 in mitochondrial membranes enhances coupling of the CI half-redox reactions, facilitating electron transfer and decreasing its net JH2O2 emitting potential (17).

Site I1 of CI does not normally produce H2O2 during forward electron flow (34) but can become a significant source under conditions that promote a high membrane potential and reduced ubiquinone pool (32), such as when electrons feed directly into the quinone pool from β-oxidation, succinate, etc., in excess of demand (49) and/or under various pathological states (50, 51). Surprisingly in liver mitochondria, E2 treatment of OVX mice in vivo increased both NADH oxidation and
JH₂O₂ production from CI. E₂ exposure of mitochondria from OVX mice in vitro also tended to increase JH₂O₂ production at site IQ but not site IF. Electron transfer through CI is thought to be limited by quinone binding and release at site IQ (52, 53). Thus, even a slight enhancement in NADH oxidation activity without a concomitant increase in quinone reduction activity could reduce the iron–sulfur centers along the peripheral arm of CI (52) and lead to higher JH₂O₂ production at the IQ site in hepatocytes.

Oxidative stress plays a key role in the pathophysiology of NAFLD (54–56) with elevated H₂O₂ production and decreased CI function thought to be primary underlying mechanisms (55, 57, 58). The surprisingly negative but relatively modest impact of E₂ replacement on both CI kinetics and CI-derived JH₂O₂ in liver mitochondria in the present study raises the possibility that E₂ therapy may promote H₂O₂ generation and potentially worsen the oxidative burden in hepatocytes, particularly with oral administration of E₂. These findings, however, are early and should be interpreted cautiously given that in vivo protein markers of cellular redox state in liver were not affected by E₂ loss and replacement. Thus, further research is needed to understand the full consequences of E₂ loss and E₂ therapy on liver mitochondrial function, especially in the context of other potential aggravating factors (i.e. high-fat diet, diabetes, etc.) to better define the risk-to-benefit ratio of ET in postmenopausal patients with NAFLD (7, 59).

**Experimental procedures**

**Reagents**

All chemicals were purchased from Sigma–Aldrich and of the highest purity available.

**Animal use**

All animal studies were approved by the East Carolina University Institutional Animal Care and Use Committee. C57BL-6N female mice were purchased from The Jackson Laboratory and housed in a 12-h light/dark cycle, temperature-controlled (22 °C) facility with free access to water and food (standard chow diet). The mice were ovariectomized at 10 weeks of age and sacrificed at 12 weeks old (OVX-2w) or...
implanted a subcutaneous micro-osmotic pump (Alzet model no. 1002) to receive an extra 2 weeks of E2 therapy (OVX-4w(E2)) or control saline solution (OVX-4w(ctl)). Water-soluble β-estradiol (Sigma catalog no. E4389) was dissolved in sterile saline, and the solution was sterilized by filtering through a 0.2-µm Millipore membrane. Each pump contained 17.5 µg of E2 (46.7 mg/g purity) in 100 µl of sterile saline, delivering ~1 µg E2/day at 0.25 µl/h. To ensure high physiological E2 plasma levels, normally cycling females in the proestrus stage of the estrus cycle (NC-Pro) were selected as the control group. Estrus cycle was monitored daily via crystal violet staining and vaginal cytology evaluation (17). Liver was collected for mitochondria isolation, and quadriceps, plantaris, soleus, and whole gastrocnemius muscles were harvested for SM mitochondria isolation.

**Mitochondrial isolation and relative specific activity of mitochondrial OXPHOS complexes**

Liver and SM were harvested at sacrifice and immediately homogenized in mitochondrial isolation medium (0.3 M sucrose, 10 mM HEPES, 1 mM EGTA) containing 1 mg/ml BSA, on ice (adapted from Ref. 60). Aliquots of isolated mitochondria were diluted to 0.8 mg/ml in hypotonic medium (25 mM K2HPO4, 5.3 mM MgCl2, pH 7.2), and further subjected to three or four freeze–thaw cycles. CI activity was determined in 5 mM Tris, 0.5 mg/ml BSA, 34 µM KCN, 0.4 µM antimycin A, pH 8, by 1) following the oxidation of NADH (0.13 mM) at 340 nm (ε340 = 6220 M⁻¹ cm⁻¹) for 3 min using 50 µM oxidized decylubiquinone (DCUox) as the electron acceptor, or 2) following the reduction of DCUox at 280 nm (ε280 = 16,000 M⁻¹ cm⁻¹) in the presence of 0.13 mM NADH. 4 µM rotenone was added in the end to measure rotenone-sensitive NADH-DCU oxidoreductase activity. Specific activities of all other individual ETS complexes, I+II, and II+III were determined spectrophotometrically as described in Refs. 17 and 61).

**Mitochondrial content**

Citrate synthase activity was measured according to specifications by Sigma catalog no. CS0720 in isolated mitochondria. OXPHOS complexes were measured in isolated mitochondria by reducing Western blotting analysis using anti-Rt/Ms total OXPHOS complex mixture (Invitrogen catalog no. 458099). Equal protein load (25 µg/well) and consistent gel transfer was verified by Ponceau S stain. The blots were imaged using IRDye 680RD donkey anti-mouse IgG antibody (Licor Biosciences catalog no. 926-68072) in a Licor IR imager and quantified using Image Studio Lite within linear range of intensity (version 5.0, Licor Bioscience).

**Mitochondrial respiration (JO2) and H2O2 emitting potential (JH2O2) measurements**

JO2 was measured in fresh isolated mitochondria using high-resolution respirometers (O2K, OROBOROS, Innsbruck, Austria), in buffer Z supplemented with 20 mM creatine monohydrate and 25 µM blebbistatin. JH2O2 was measured using the Amplex UltraRed/horseradish peroxidase fluorescence system in FluoroMax/Fluorolog spectrofluorometers (HORIBA Jobin Yvon). All JH2O2 experiments were performed in buffer Z supplemented with 10 µM Amplex UltraRed (Invitrogen), and 20 units/ml copper–zinc SOD (Sigma). For CI-targeted experiments in freeze-fractioned mitochondria, assays were performed in the same way, in the presence of 5 mM Tris, 0.5 mg/ml BSA, 24 µM CN, 0.4 µM antimycin A, pH 8, and 50 µM DCUox as the electron acceptor. The addition of NADH (0.13 mM) as the electron donor started the reaction.

**Measurements of cellular and mitochondrial redox state**

Adapting the protocol from Refs. 62 and 63, and as performed in Ref. 17, GSH and GSSG were measured by HPLC. GSH was measured as its conjugate with N-ethylmaleimide (NEM). The liver was homogenized immediately after collection in buffer containing NEM, and GS-NEM conjugate was measured by UV detection, in which two diastereomers are visible as equally sized peaks in the chromatogram. GSSG was measured in a parallel sample of the muscle homogenate using fluorescent detection of GSSG binding to o-phthalaldehyde at pH 12. Samples selected to measure GSH/GSSG by HPLC were also utilized for Western blotting analysis of Prx oxidation. Reduced, oxidized-dimer, and oxidized-decamer forms of peroxiredoxins (Prx2, cytosolic; and Prx3, mitochondrial) were measured in liver homogenates by standard nonreducing Western blotting analysis as described in Ref. 28. Ponceau S stain was used to verify equal protein load (45 µg/well) and consistent gel transfer. Anti-Prx2 (AbCam, ab109367) and anti-Prx3 (AbFrontier, LF-MA0044) were used for protein detection. The blots were imaged using a Licor IR imager and quantified using Image Studio Lite (version 5.0, Licor Bioscience). As a positive control, samples were treated with β-mercaptoethanol (20× dilution) to verify oxidized and reduced Prx bands. Oxidized decamer (250 kDa), oxidized dimer (45 kDa), and reduced (22–25 kDa) forms of Prx2 and Prx3 were quantified and presented as fold difference relative to NC-Pro.

**GSH reductase activity**

Determined in liver homogenates in buffer 100 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% Tween, pH 8.35, as described in a Cayman chemical kit (catalog no. 703202).

**Statistical analyses**

The data are presented as means ± S.E. Comparisons between two groups were performed by independent (or paired, when applicable) Student’s t tests. Comparisons between four groups were performed with one-way ANOVA, followed by Sidak’s multiple comparisons test (versus NC-Pro). Graph Pad Prism 7 was used for statistical tests and data presentation. Statistical significance was set at p < 0.05. The p values are provided in the figure legends.
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