Reproductive Sciences
2019, Vol. 26(1) 114-127
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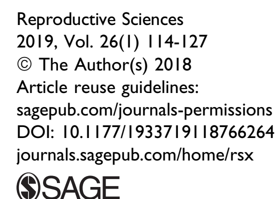

Changes in Metabolism, Mitochondrial Function, and Oxidative Stress Between Female Rats Under Nonreproductive and 3 Reproductive Conditions

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Abstract

Women who do not lactate display increased incidence of obesity, type II diabetes, and cancer. Stuebe and Rich-Edwards proposed that these effects occur because physiological changes that ensue during pregnancy are not reversed without lactation. To empirically test this hypothesis, we compared markers of metabolism, mitochondrial function, and oxidative stress between 4 groups of Sprague-Dawley rats: (1) nonreproductive (NR) rats, (2) rats killed at day 20 of gestation, (3) rats that gave birth but were not allowed to suckle their pups (nonlactating), and (4) rats that suckled their young for 14 days. Nonlactating females displayed higher body fat compared to all other groups. Peroxisome proliferator-activated receptor δ (PPARδ) in skeletal muscle and white adipose tissue of nonlactating rats was lower than the other groups. The PPARδ is associated with lipid metabolism suggesting that the higher fat mass in nonlactating females was not associated with the retention of a physiological state that was set during pregnancy but instead an independent drop in PPARδ. Relative mitochondrial respiratory function and complex activity in the liver and skeletal muscle of nonlactating mice were not predictive of higher body mass, and measures of oxidative stress displayed minimal variation between groups.

Keywords

lactation, mitochondria, oxidative stress

Introduction

Women who do not breast-feed their young have increased incidence of obesity and type II diabetes relative to women who do breast-feed.[1,2] While factors involved in the onset of metabolic disease are multifactorial, the potential physiological mechanisms involved in disposing women who do not breast-feed to higher incidence of metabolic disease are largely unknown. During pregnancy, the female's body supports fetal growth and undergoes physiological adaptations in preparation for lactation. An increase in maternal insulin production facilitates glucose transport to the fetus,[3] and a concordant rise in lipid storage increases visceral adiposity.[4] At the onset of lactation, a metabolic shift occurs that redirects energy substrates, including stored lipids, to the mammary glands for use in milk synthesis. Stuebe and Rich-Edwards proposed that health disparities between women who breast-feed and those who do not may occur because the metabolic changes that promote the production of milk during lactation reverse, or reset, the changes to a women's metabolism that occur during pregnancy.[2] Thus, without the metabolic shift associated with lactation, a female's body may remain in a state that promotes greater circulating glucose and lipid storage.[2]

Mitochondria support the energy demands of the cell and play a vital role in regulating cellular metabolism.[5,6] Tissue-specific modulation of mitochondrial function is likely to underlie the metabolic shifts that organ/tissues experience during reproduction. Indeed, markers of mitochondrial biogenesis and lipid metabolism (ie, peroxisome proliferator-activated receptor alpha [PPAR-α], PPAR gamma coactivator 1 alpha [PGC-1α], and PPAR gamma coactivator 1 beta [PGC-1β]) have previously been demonstrated to be downregulated during lactation in skeletal muscle and liver in mice.[7] Furthermore, the mitochondrial uncoupling protein UCP3 has also been observed to decrease in skeletal muscle during lactation.[8,9] These cellular adaptations support the idea that energy

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resources are allocated away from tissues less important in producing energy for the offspring toward those that are.[10]

Variance in mitochondrial performance also contributes to the production of reactive oxygen species (ROS) when electrons escape the electron transport system. An imbalance between ROS production and the counteracting antioxidant system allows ROS to interact with and cause damage to proteins, lipids, and DNA.[11] This imbalance is referred to as oxidative stress. Change in relative oxidative stress is a reported consequence of reproduction.[12,13] Combined with a change in mitochondrial respiratory performance, an increase in oxidative stress has also been purported to contribute to metabolic disease, cancer, senescence, and aging,[14] Given that mitochondrial performance and oxidative stress both appear to vary with reproduction and with metabolic disease and cancer,[15] evaluating mitochondrial respiratory function and its regulators along with oxidative stress between reproductive females who suckle their pups and females who do not suckle is likely to provide valuable insight into why women who do not breast-feed are at increased risk of health disparities.

The goal of this investigation was to characterize differences in metabolism, mitochondrial function, and oxidative stress in rats that were nonreproductive (NR), pregnant, lactating, and those who gave birth but did not suckle their young. Following the observations of Stuebe and Rich-Edwards[2] in humans, we utilized a rat model to detect any early metabolic and mitochondrial differences associated with reproduction and lactation. Specifically, we predicted that females who did not lactate would display metabolic, mitochondrial, and oxidative stress profiles that are more comparable to pregnant females than lactating or NR females. These data will provide valuable insight into early differences between these groups that set the stage for subsequent decline in a female's health.

Methods

Animal Husbandry

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee and were carried under the guidelines of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Ten-week-old Sprague-Dawley rats were obtained from Envigo. Animals were acclimated with their diet and facility for 10 days prior to experimental start. Rats were housed under standard laboratory conditions (46 $\times$ 25 $\times$ 20 cm boxes, 12 light–12 dark cycle, 22°C, 50% relative humidity [RH]) and given ad libitum access to food (Teklad Global Diet 2018) and water. Animals ($n = 8$ per group) were randomly assigned to 1 of 4 treatment groups: (1) NR animals, (2) animals that became pregnant and were killed at day 20 of gestation (P), (3) animals that became pregnant, gave birth, but had their pups removed within 12 hours of birth and thus did not lactate (NL), and (4) animals that became pregnant, gave birth, and suckled their young to peak lactation at 14 days (L). Female rats were paired in boxes with same-group counterpart, but animals in groups that underwent mating were separated during late pregnancy. The NR animals were age matched to females in the other groups and killed at a time that corresponded to P animals at 20th day of pregnancy. The L animals had their litter size adjusted to 8 on the day of parturition. The NL and L animals were age matched and killed at a time that corresponded to the 14 days of lactation in PL animals.

Blood Collection and Analysis

Rats were fasted 4 hours prior to blood collection. Animals were anesthetized using isoflurane vapors, and body mass was quickly recorded. The anesthetized animals were then decapitated, and blood was collected, allowed to clot, and then centrifuged. Following centrifugation, the serum was frozen at −80°C for subsequent analyses. Serum glucose (STA-680, Cell Biolabs, San Diego, California) and nonesterified fatty acids (NEFA; STA-618, Cell Biolabs) were quantified using the manufacturer's specifications.

Tissue Collection and Handling

After the decapitation, the following tissues were excised and weighed: liver, triceps surae ("calf" muscle), retroperitoneal white adipose tissue (RetroP WAT), and perirenal white adipose tissue (PR WAT) pads. After the mass of each tissue was recorded, a sample from calf skeletal muscle and liver was used for mitochondrial isolation, and the remainder of tissues was frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

Mitochondrial Isolation

Mitochondrial isolations for muscle were performed as described previously.[16] Excised muscles (~750 mg) were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mmol/L KCl, 40 mmol/L Tris HCl, 10 mmol/L Tris base, 1 mmol/L $MgSO_4$, 0.1 mmol/L EDTA, 0.2 mmol/L ATP, and 0.2% [wt/vol] free fatty acid bovine serum albumin [BSA], pH 7.40). Muscles were minced with scissors, and the mince was homogenized for 15 seconds with a polytron. Protease (trypsin) was added (5 mg/g wet muscle), and the digested mince was mixed continually for 7 minutes. Digestion was terminated by the addition of an equal volume of solution I. The homogenate was centrifuged at 500*g* for 10 minutes at 4°C, and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3500*g* for 10 minutes. The supernatant was discarded, and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3500*g* for 10 minutes. The supernatant was again discarded, and the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3500*g* for 10 minutes. The final mitochondrial pellet was suspended in 250

μL of a solution containing 220 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L Tris HCl, and 1 mmol/L EGTA, pH 7.40. Mitochondria from liver were isolated as described previously.[15] Briefly, liver (~750 mg) was weighed and placed in 10 volumes of solution III (250 mmol/L sucrose, 5 mmol/L HEPES, and 1 mmol/L EGTA), minced with scissors, and the mince was homogenized with a Potter-Elvehjem PTFE pestle and glass tube (2 passes). The homogenate was centrifuged at 500*g* for 10 minutes at 4°C. The supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3500*g* for 10 minutes. The supernatant was discarded, and the mitochondrial pellet was resuspended in solution III. The suspension was centrifuged at 3500*g* for 10 minutes. The final mitochondrial pellet was suspended in 250 μL of a solution containing (in mmol/L) 220 mannitol, 70 sucrose, 10 Tris HCl, and 1 EGTA, pH 7.40.

Isolated Mitochondrial Oxidative Phosphorylation

Mitochondrial oxygen consumption was measured for liver and muscle tissue as described.[16] Briefly, mitochondrial oxygen consumption was measured polarographically in a respiration chamber (Hansatech Instruments, Lynn, United Kingdom). Isolated mitochondria (20 μL) were incubated with 1 mL of respiration buffer (100 mmol/L KCL, 50 mmol/L MOPS, 10 mmol/L KH_2PO_4, 20 mmol/L glucose, 10 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 0.2% fatty acid free BSA; pH = 7.0) at 37°C in a respiratory chamber with continuous stirring. For state 3 respiration, 2 mmol/L pyruvate and 2 mmol/L malate (complex I substrates) or 5 mmol/L succinate (complex II substrate) were used in the presence of 0.25 mmol/L adenosine diphosphate (ADP), and state 4 respiration was recorded following the phosphorylation of ADP. Respiratory control ratio (RCR) was calculated as state 3/state 4 oxygen consumption. Respiration values were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the samples.

Mitochondrial Oxidant Emission

Oxidant emission by mitochondria was determined using the oxidation of the fluorogenic indicator Amplex Red (Molecular Probes, Eugene, Oregon) in the presence of horseradish peroxidase.[17] The assay was performed at 37°C in 96-well plates using succinate as the substrate. Specifically, this assay was developed based on the concept that horseradish peroxidase catalyzes the hydrogen peroxide-dependent oxidation of nonfluorescent Amplex Red to fluorescent Resorufin Red. Resorufin Red formation was monitored at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a multiwell plate reader fluorometer (Synergy H1, BioTek, Winooski, Vermont). We recorded the level of Resorufin Red formation, and hydrogen peroxide production was calculated with a standard curve.

Enzymatic Assays for Electron Transport Chain Complex Activity

Complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition.[18] Complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction.[18] Complex III (ubiquinol cytochrome *c* oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from cytochrome *c* reduction.[18] Complex IV (cytochrome *c* oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation.[18] Specificity of complex IV activity was determined by monitoring changes in absorbance in the presence of KCN.[18] Citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5′-dithiobis-2-nitrobenzoic acid reduction.[18] Enzyme activities were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the cell samples.

Protein Abundance

The relative concentration of proteins was quantified by Western blot analysis.[17] To accomplish this, tissue was homogenized 1:10 (wt/vol) in 5 mmol/L Tris HCl (pH 7.5), 5 mmol/L EDTA (pH 8.0), and protease inhibitor cocktail (14224-396, VWR, Radnor, Pennsylvania) and was centrifuged at 1500*g* for 10 minutes at 4°C. Protein content of the supernatant was quantified by the method of Bradford.[19] Proteins were separated by polyacrylamide gel electrophoresis via 4% to 20% polyacrylamide gels (Bio-Rad, Hercules, California). After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific sites were blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween-20 and 5% nonfat milk. Membranes were then incubated overnight at 4°C with primary antibodies purchased from GeneTex (Irvine, California) directed against PPARα (GTX101096, 1:1000), PPAR delta (PPARδ, GTX113250, 1:2000), PGC-1α (GTX37356, 1:1000), superoxide dismutase 1 (SOD1, GTX100554 1:2000), superoxide dismutase 2 (SOD2, GTX116093, 1:2000), catalase (CAT, GTX110704, 1:2000), and glutathione peroxidase (GPX, GTX116040, 1:2000). Following incubation with primary antibodies, membranes were washed (5 minutes × 3) with PBS-Tween and then incubated with secondary antibodies for 1 hour at room temperature. After washing (5 minutes × 3), a chemiluminescent system was used to detect labeled proteins (GE Healthcare, Buckinghamshire, United Kingdom). Images of the membranes were captured and analyzed using the ChemiDoc-It2 Imaging System (UVP, LLC, Upland, California). Protein expression was normalized to Ponceau staining.

Assessment of Indices of Oxidative Damage

To determine the relative amount oxidative damage, we measured protein oxidation and lipid peroxidation. Lipid

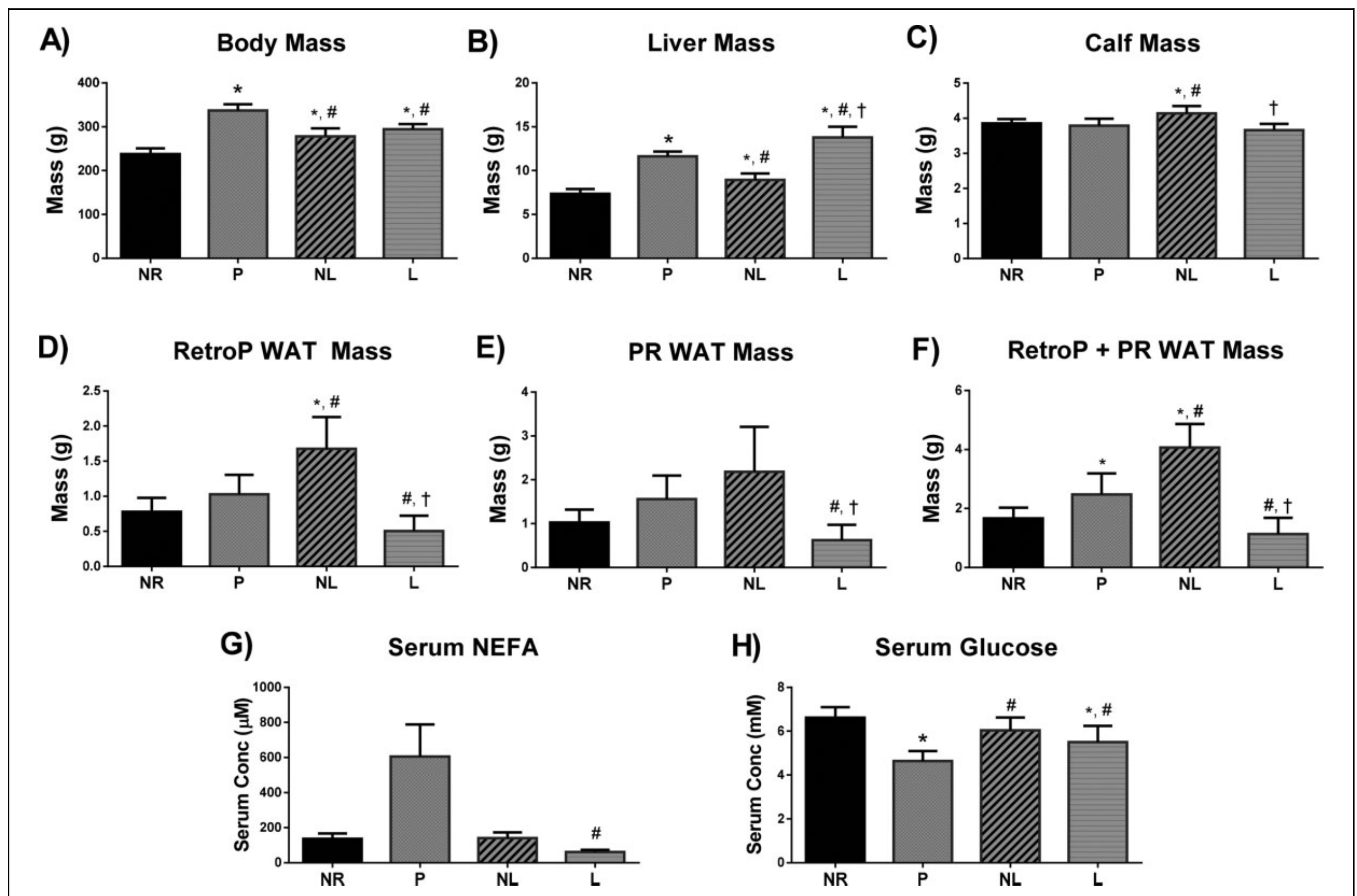

Figure 1. Body mass, tissue mass, and serum metabolites for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were killed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). (A) Body mass, (B) liver mass, (C) mass of both rear triceps surae (calf muscle mass), (D) retroperitoneal white adipose tissue (RetroP WAT) mass, (E) perirenal (PR) WAT mass, (F) combined mass of RetroP and PR WAT, (G) serum concentration of nonesterified fatty acids (NEFA), and (H) serum concentration of glucose. Data shown are mean ± standard deviation (SD). * Different from NR ($P < .05$), # different from P ($P < .05$), and † different from NL ($P < .05$).

peroxidation was assessed by determining 4-hydroxynoneal (4-HNE; *trans*-4-hydroxy-2-nonenal, $C_9H_{16}O_2$) expression via Western blotting. Primary antibody for 4-HNE was purchased from Abcam (ab46545; 1:1000 dilution; Cambridge, Massachusetts). Protein oxidation was measured by comparing relative expression of protein carbonyls using a commercially available kit (Oxyblot Protein Oxidation Detection Kit; Intergen, Purchase, New York) via Western blotting as described by the manufacturer's instructions.

Statistics

Comparison between groups for each dependent variable was made by a 1-way analysis of variance (ANOVA), with a Tukey post hoc test being used to determine significant differences between groups when the ANOVA indicated statistical significance. However, in the case of PR WAT mass, serum NEFA, state 3 succinate liver, complex I RCR muscle, state 3 succinate muscle, SOD2 liver, CAT liver, PGC-1a muscle, and 4-HNE RetroP WAT, the Brown-Forsythe test was significant, and thus, the Kruskal-Wallis test was performed followed by the Dunn for multiple comparisons post hoc. Data are presented as means ± standard deviation, and significance was established at $P < .05$.

Results

Body and Tissue Mass

The transition into pregnancy and lactation periods is matched with large variations in mass. Body mass was the highest in pregnant animals compared to all groups. Body mass of both nonlactating and lactating animals dropped after parturition but was still higher compared to NR animals, $F_{(3,\ 27)} = 59.6$, $P < .05$ (Figure 1A). Liver mass was increased in pregnant animals compared to NR animals. Following parturition, liver mass of nonlactating animals decreased, but the liver mass of lactating animals increased and had the highest mass of all 4 groups, $F_{(3,\ 26)} = 87.2$, $P < .05$ (Figure 1B). The combined mass of the triceps surae calf muscle (henceforth skeletal

Table 1. Isolated Liver Mitochondria Respiration.[a]

Group	State 3 P/M	State 4 P/M	RCR P/M	State 3 Suc	State 4 Suc	RCR Suc
NR	156.3 ± 22.8	31.2 ± 5.2	4.6 ± 0.4	529.5 ± 85.1	86.4 ± 5.8	6.0 ± 0.6
P	131.6 ± 8.3	34.8 ± 2.1	3.8 ± 0.4[b]	466.0 ± 51.1	93.3 ± 5.3	5.2 ± 0.4[b]
NL	166.4 ± 19.2[c]	34.6 ± 7.3	4.5 ± 0.3[c]	479.7 ± 34.0	87.4 ± 15.2	5.7 ± 0.4
L	161.1 ± 19.2[c]	30.9 ± 3.3	5.1 ± 0.5[c]	483.8 ± 23.0	83.0 ± 7.6	5.7 ± 0.5

Abbreviations: NR, nonreproductive rats; P, rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation; NL, rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups; L, rats that were allowed to mate, became pregnant, and suckled their young for 14 days; P/M, pyruvate and malate; suc, succinate; RCR, respiratory control ratio.
[a]Units for State 3 and State 4 are pmol O_2/min/citrate synthase unit. Data shown are mean ± SD.
[b]Different from NR ($P < .05$).
[c]Different from P ($P < .05$).

Table 2. Isolated Skeletal Muscle Mitochondria Respiration.[a]

Group	State 3 P/M	State 4 P/M	RCR P/M	State 3 Suc	State 4 Suc	RCR Suc
NR	96.6 ± 21.0	11.6 ± 3.2	8.6 ± 2.1	81.8 ± 5.7	26.3 ± 2.1	3.1 ± 0.2
P	103.0 ± 14.8	11.3 ± 1.4	8.5 ± 0.6	86.2 ± 4.0	28.3 ± 2.6	2.9 ± 0.1
NL	104.2 ± 15.7	12.4 ± 1.8	8.4 ± 0.7	87.0 ± 11.8	27.4 ± 3.5	3.1 ± 0.1
L	99.9 ± 23.1	11.2 ± 2.6	8.8 ± 0.8	79.7 ± 9.1	24.3 ± 2.5	3.3 ± 0.1[b]

Abbreviations: NR, nonreproductive rats; P, rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation; NL, rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups; L, rats that were allowed to mate, became pregnant, and suckled their young for 14 days; P/M, pyruvate and malate; suc, succinate; RCR, respiratory control ratio.
[a]Units for State 3 and State 4 are pmol O_2/min/citrate synthase unit. Data shown are mean ± SD.
[b]Different from P ($P < .05$).

muscle) was the highest in nonlactating animals compared to all groups, $F_{(3, 26)} = 9.8$, $P < .05$ (Figure 1C). RetroP WAT mass was higher in nonlactating animals compared to all groups, and lactating animals had lower RetroP, $F_{(3, 25)} = 20.1$, $P < .05$, and PR WAT (KW = 14.4, $P < .05$) mass compared to pregnant and nonlactating animals (Figure 1D and E). The combined mass of RetroP and PR was highest in nonlactating animals while also being higher in pregnant animals compared to NR and lactating animals, $F_{(3, 24)} = 28.5$, $P < .05$ (Figure 1F).

Serum Glucose and NEFA Concentrations

Serum glucose concentration was lower in pregnant animals compared to all groups ($P < .05$). Lactating animals also experienced lower serum glucose compared to NR animals, $F_{(3, 27)} = 16.8$, $P < .05$ (Figure 1G). Pregnant animals had high serum NEFA, albeit not significant, and lactating animals had lower free fatty acids compared to pregnant animals (KW = 22.8, $P < .05$; Figure 1H).

Mitochondrial Function and Oxidant Emission

The RCR of liver mitochondria with complex I substrate was decreased in pregnant animals compared to all groups, $F_{(3, 25)} = 10.4$, $P < .05$, and had lower RCR with complex II substrate compared to NR animals ($F_{3,27} = 2.7$, $P < .05$; Table 1). No differences were detected between groups in RCR in mitochondria isolated from skeletal muscle with complex I substrate (KW = 0.85, $P > .05$). However, RCR was higher in skeletal muscle mitochondria isolated from lactating animals compared to pregnant animals when using complex II substrate, $F_{(3, 26)} = 3.4$, $P < .05$, (Table 2). The ROS are a potential by-product that can occur at complex I and III sites in mitochondria during respiration.[20] Oxidant emission in isolated liver mitochondria was lower in nonlactating animals compared to pregnant animals (NR = 238.6 ± 52.1; P = 285.6 ± 95.3; NL = 181.8 ± 46.1; L = 264.0 ± 29.5 H_2O_2/min/CS unit), $F_{(3, 21)} = 3.1$, $P < .05$ (Figure 2G). No differences were detected in oxidant emission from skeletal muscle mitochondria (NR = 13.9 ± 5.9; P = 12.6 ± 4.7; NL = 10.9 ± 5.8; L = 16.7 ± 6.9 H_2O_2/min/CS unit), $F_{(3, 23)} = 1.0$, $P > .05$.

Mitochondrial Complex Activity

Enzymatic activity of liver and skeletal muscle mitochondria are shown in Table 3. Complex I was higher in liver of nonlactating animals compared to all groups, $F_{(3, 26)} = 6.2$, $P < .05$. Conversely, nonlactating and lactating animals both had lower liver complex II activity compared to NR and pregnant animals, $F_{(3, 24)} = 15.9$, $P < .05$. No differences were detected for complex III, $F_{(3, 27)} = 1.6$, $P > .05$, and IV, $F_{(3, 25)} = 0.94$, $P > .05$, activity in liver mitochondria. Complex I activity of skeletal muscle mitochondria was lower in lactating animals compared to pregnant animals, $F_{(3, 27)} = 5.8$, $P < .05$. Additionally, complex III activity in skeletal muscle was higher in nonlactating and lactating animals compared to NR animals, $F_{(3, 26)} = 7.2$, $P < .05$. No differences were detected at

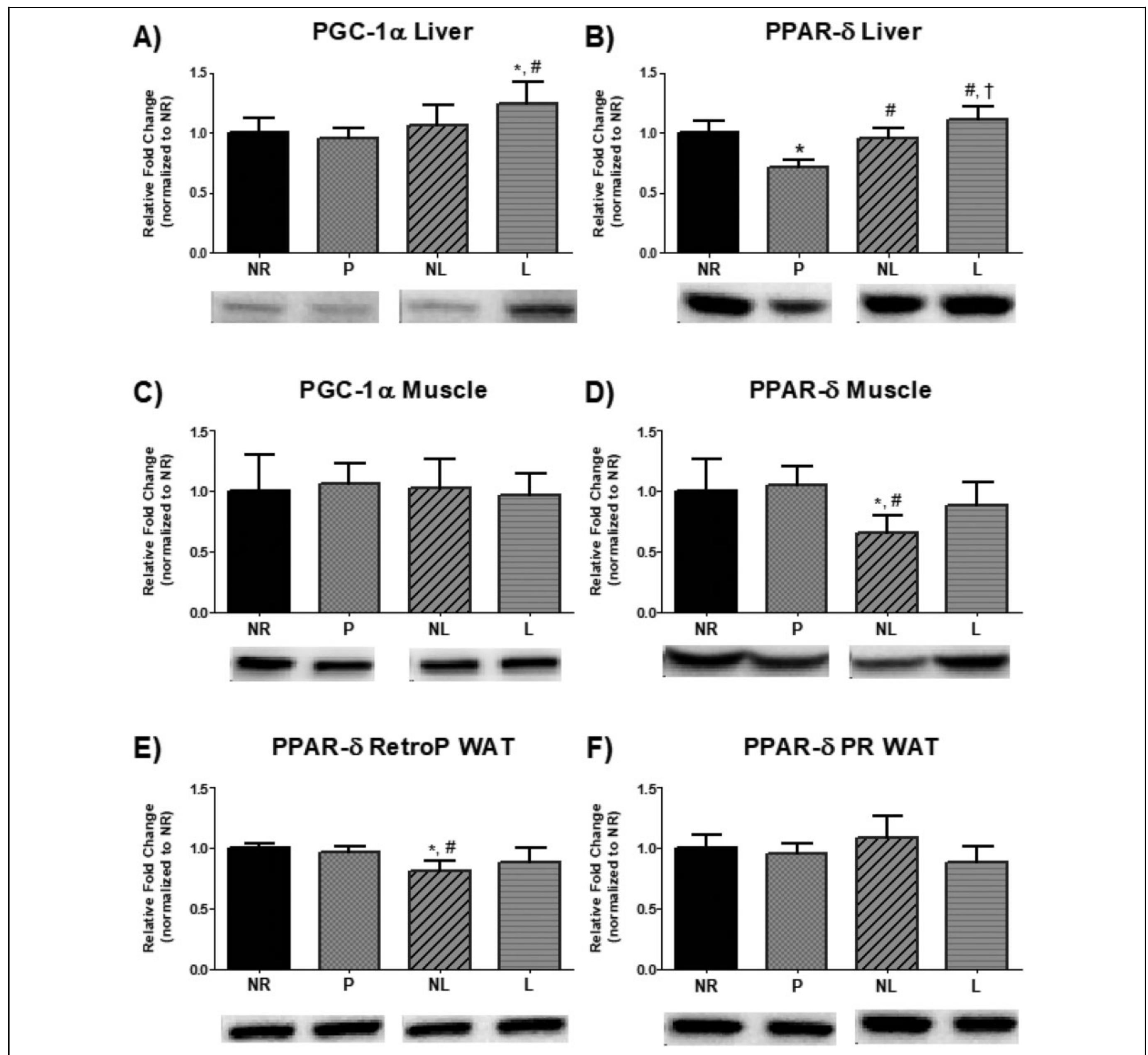

Figure 2. Markers of metabolism in liver, skeletal muscle, and white adipose tissue (WAT) for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include (A) PGC-1α and (B) PPARδ protein levels in liver. (C) PGC-1α and (D) PPARδ protein levels in skeletal muscle. Also, PPARδ protein levels in (E) retroperitneal (RetroP) and (F) perirenal (PR) WAT are shown. Representative blots are shown under the graphs. Data shown are mean ± standard deviation (SD). * Different from NR ($P < .05$), # different from P ($P < .05$), and † different from NL ($P < .05$). PGC-1α indicates PPAR gamma coactivator 1 alpha; PPARδ, peroxisome proliferator-activated receptor delta.

complex II, $F_{(3,\ 24)} = 0.89$, $P > .05$, and IV, $F_{(3,\ 25)} = 1.5$, $P > .05$, in mitochondria isolated from skeletal muscle.

Markers of Metabolism

Levels of PGC-1α protein were higher in the liver of lactating animals compared to NR and pregnant animals, $F_{(3,\ 25)} = 5.5$, $P < .05$ (Figure 2A). Liver PPARδ protein expression was lower in pregnant animals compared to all groups and was higher in lactating animals compared to nonlactating animals, $F_{(3,\ 28)} = 26.1$, $P < .05$ (Figure 2B). The PPARδ protein levels were lower in nonlactating animals compared to NR and pregnant animals in skeletal muscle, $F_{(3,\ 24)} = 4.7$, $P < .05$, and in RetroP WAT, $F_{(3,\ 23)} = 8.1$, $P < 0.05$ (Figure 2D and E). No

Table 3. Isolated Liver and Muscle Mitochondria Complex Activity.[a]

Group	Complex I	Complex II	Complex III	Complex IV
Liver				
NR	0.29 ± 0.03	0.08 ± 0.01	0.66 ± 0.11	8.0 ± 0.45
P	0.29 ± 0.04	0.089 ± 0.01	0.55 ± 0.07	8.1 ± 0.35
NL	0.38 ± 0.05[bc]	0.059 ± 0.01[bc]	0.59 ± 0.13	7.9 ± 0.49
L	0.28 ± 0.05[d]	0.045 ± 0.01[bc]	0.64 ± 0.08	8.3 ± 0.46
Muscle				
NR	0.12 ± 0.01	0.023 ± 0.006	0.17 ± 0.05	2.0 ± 0.35
P	0.14 ± 0.01	0.021 ± 0.003	0.23 ± 0.04	2.2 ± 0.20
NL	0.11 ± 0.02	0.019 ± 0.005	0.24 ± 0.03[b]	2.2 ± 0.18
L	0.09 ± 0.02[c]	0.019 ± 0.004	0.28 ± 0.03[b]	2.2 ± 0.27

Abbreviations: NR, nonreproductive rats; P, rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation; NL, rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups; L, rats that were allowed to mate, became pregnant, and suckled their young for 14 days.
[a]Units are in μM/min/citrate synthase unit. Data shown are mean ± SD.
[b]Different from NR ($P < .05$).
[c]Different from P ($P < .05$)
[d]Different from NL ($P < .05$).

differences were detected for PGC-1α protein levels in skeletal muscle (KW = 0.93, $P > .05$; Figure 2C) or PPARδ in PR WAT, $F_{(3, 25)} = 2.7$, $P > .05$ (Figure 2F).

Markers of Oxidative Stress

The GPX protein levels were lower in the liver of lactating animals compared to pregnant and nonlactating animals, $F_{3,27} = 5.3$, $P < .05$. No differences were detected in antioxidant protein expression in liver of SOD2 (KW = 7.8, $P > .05$), SOD1, $F_{(3, 27)} = 1.1$, $P > .05$, and CAT (KW = 2.1, $P > .05$). Additionally, in the liver, no differences were detected in markers of lipid peroxidation, 4-HNE, $F_{(3, 27)} = 0.02$, $P > .05$, or protein oxidation, oxyblot, $F_{(3, 24)} = 0.83$, $P > .05$ (Figure 3). The SOD2 protein levels were higher in skeletal muscle of nonlactating and lactating animals compared to pregnant and NR animals, $F_{(3, 22)} = 7.5$, $P < .05$. Additionally, 4-HNE expression was higher in skeletal muscle of nonlactating animals compared to pregnant animals, $F_{(3, 26)} = 3.6$, $P < .05$. No differences were detected in antioxidant protein expression of SOD1, $F_{(3, 24)} = 0.34$, $P > .05$; CAT, $F_{(3, 27)} = 1.2$; GPX, $F_{(3, 24)} = 0.85$, $P > .05$; or oxyblot, $F_{(3, 24)} = 1.1$, $P > .05$, in skeletal muscle (Figure 4). In RetroP WAT, SOD1 protein expression was decreased in nonlactating animals compared to pregnant animals, $F_{3,25} = 4.9$, $P < .05$, and CAT was decreased in lactating animals compared to pregnant and nonlactating animals, $F_{(3, 23)} = 4.5$, $P < .05$. No differences were detected in SOD2, $F_{(3, 23)} = 1.9$, $P > .05$; GPX, $F_{(3, 27)} = 0.90$, $P > .05$; or 4-HNE, KW = 5.5, $P > .05$, in RetroP WAT (Figure 5). In PR WAT, SOD2 protein levels were decreased in lactating animals compared to NR and nonlactating animals, $F_{(3, 25)} = 4.7$, $P < .05$, and CAT protein levels were decreased in lactating animals compared to pregnant and nonlactating animals, $F_{(3, 25)} = 6.6$, $P < .05$ (Figure 6).

Discussion

Here, we report the alterations in mitochondrial function, oxidative stress, and markers of metabolism in female rats that occur across pregnancy and a period of lactation relative to each other and relative to females who did not suckle their young or did not reproduce. We found that markers of metabolism of nonlactating females commonly differed from lactating females, suggesting that adaptations occur in the absence of lactation. While it has been proposed that health disparities between women who suckle their young and those that do not may be due to an inability to revert from the metabolic milieu established during pregnancy,[2] our results show that nonlactating females were not consistently similar to either pregnant or NR females, indicating that the changes in physiology that occur when a female doesn't lactate are neither associated with a failure to adjust from a pregnancy state nor are they associated with a return to a NR state. Instead, we found that nonlactating females displayed unique patterns of PPARδ protein expression in skeletal muscle and WAT. A detail discussion of our findings follows.

Pregnancy and Lactation Result in Body and Organ Mass and Blood Metabolite Differences

Epidemiological data suggest that women who give birth but do not breast-feed display a greater probably of developing obesity and type II diabetes than women who do breast-feed. In this study, mothers who had their offspring removed within 12-hour of birth (ie, nonlactating females) resulted in higher fat mass 2 weeks postpartum. While we found no immediate differences in body mass between female rats that suckled their young and those that did not, we found that nonlactating females carried more visceral adipose tissue than any other group, while the lactating group had a significantly heavier liver (Figure 1). Given that adipose accumulates during pregnancy in preparation for lactation, this finding lends some support to the idea that processes which upregulate adipose deposition may not be "turned off" if females do not sustain lactation. Interestingly, stored nutrient reserves may not be the only factor that can influence the lactating animal, and investigators reported that the capacity to dissipate heat plays a role in the maximum energy intake and the reproductive performance in female mice.[21] Additionally, Moore and collaborators[4,22] described comparable differences in body fat 21 days following the cessation of lactation, with nonlactating females displaying higher absolute fat mass and a greater number of visceral adipocytes than females that did lactate. Unlike visceral fat, serum NEFA and glucose did not display patterns indicative of later health disparity. Serum NEFA dropped to NR levels within 2 weeks of parturition for both lactating and nonlactating females. Serum glucose reached NR levels within 2 weeks of parturition in nonlactating rats; however, female rats that sustained lactation had lower serum glucose than NR rats after this period (Figure 1).

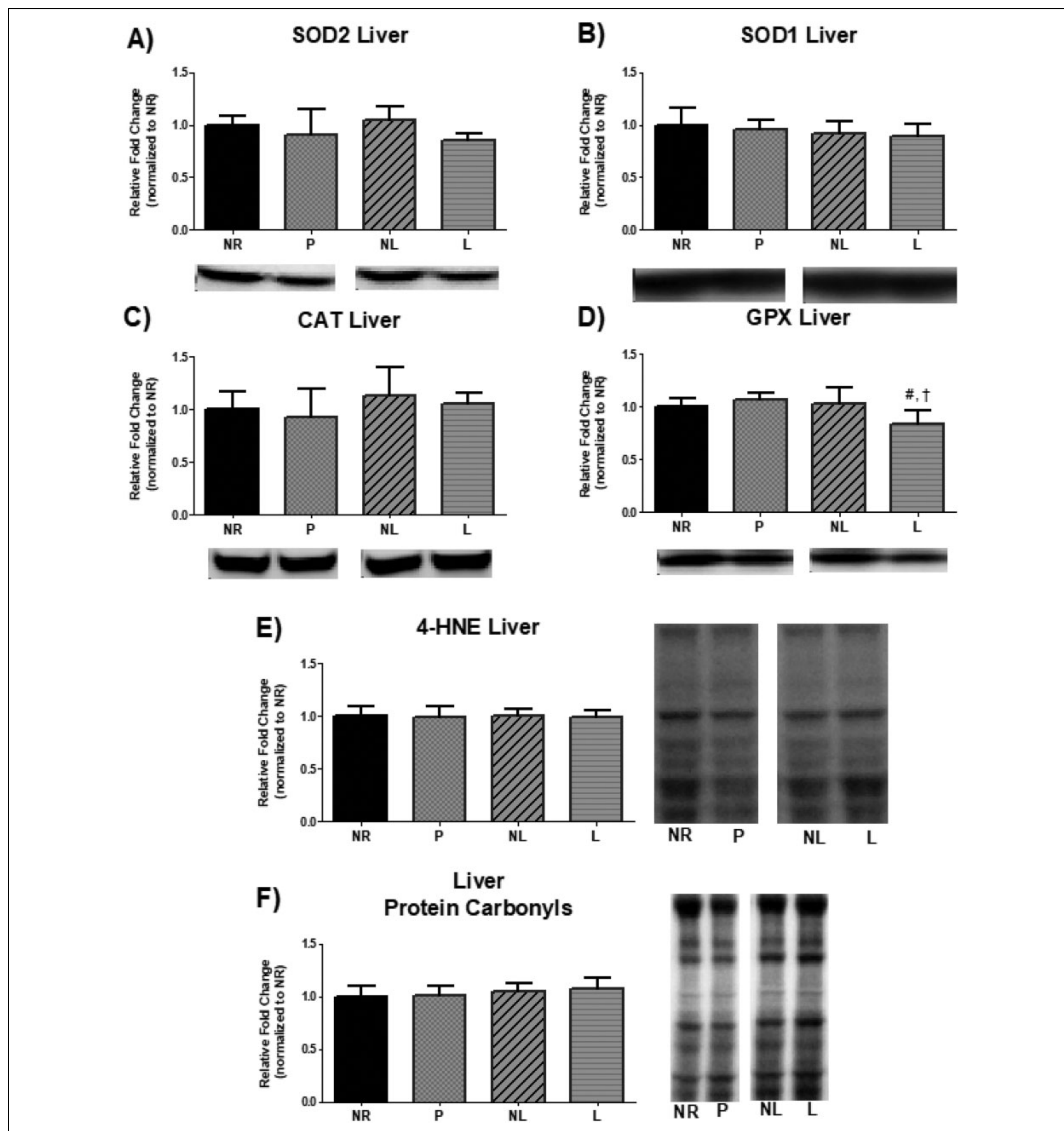

Figure 3. Markers of oxidative stress in liver for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were killed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants (A) SOD2, (B) SOD1, (C) CAT, and (D) GPX. In addition, data include markers of oxidative damage including (E) lipid peroxidation determined by 4-HNE, and (F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean $\pm$ SD. # Different from P ($P < .05$), and † different from NL ($P < .05$). SOD indicates superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

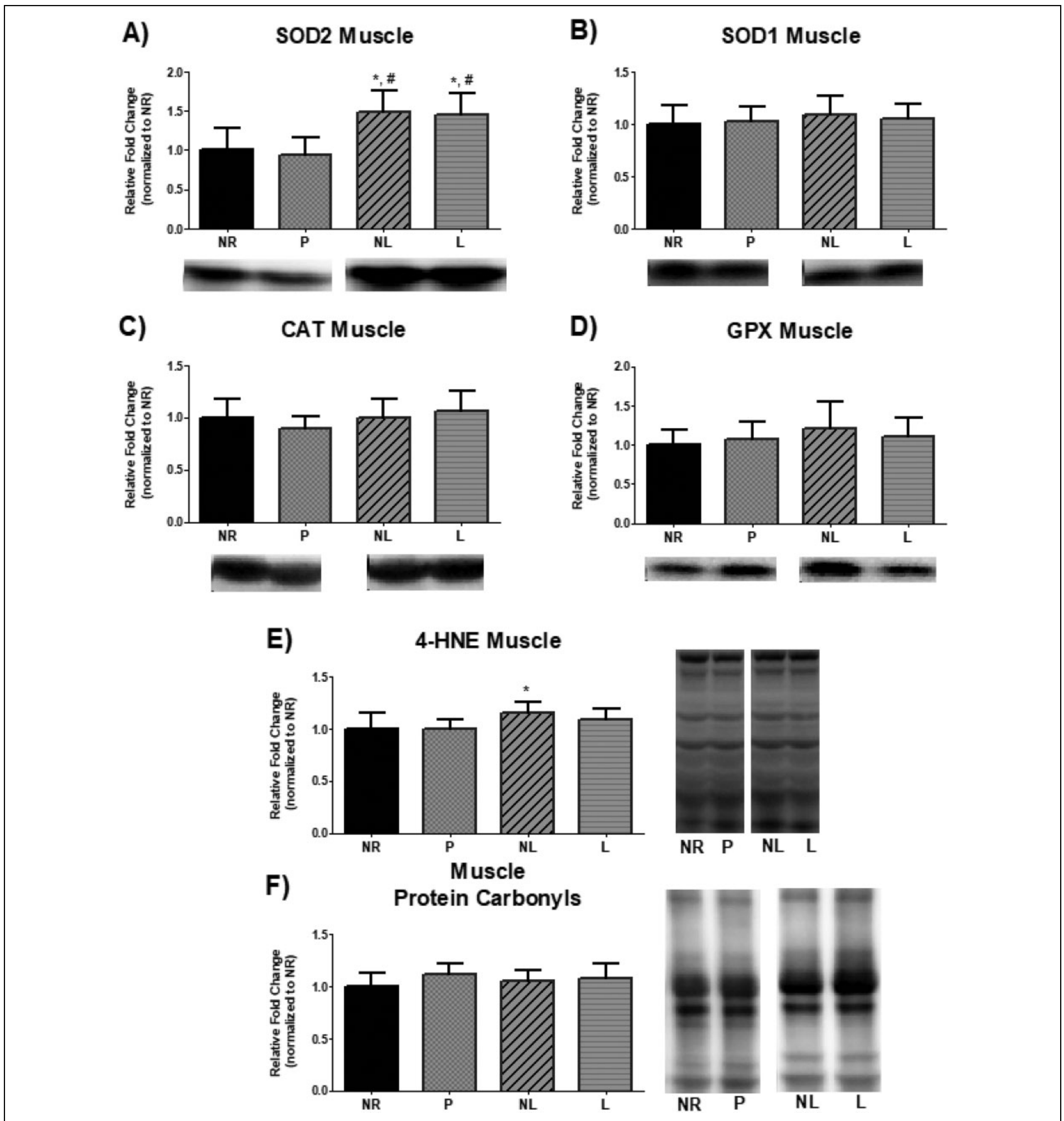

Figure 4. Markers of oxidative stress in skeletal muscle for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants (A) SOD2, (B) SOD1, (C) CAT, and (D) GPX. In addition, data include markers of oxidative damage including (E) lipid peroxidation determined by 4-HNE, and (F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean $\pm$ standard deviation (SD). * Different from NR ($P < .05$), and # different from P ($P < .05$). SOD indicates superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

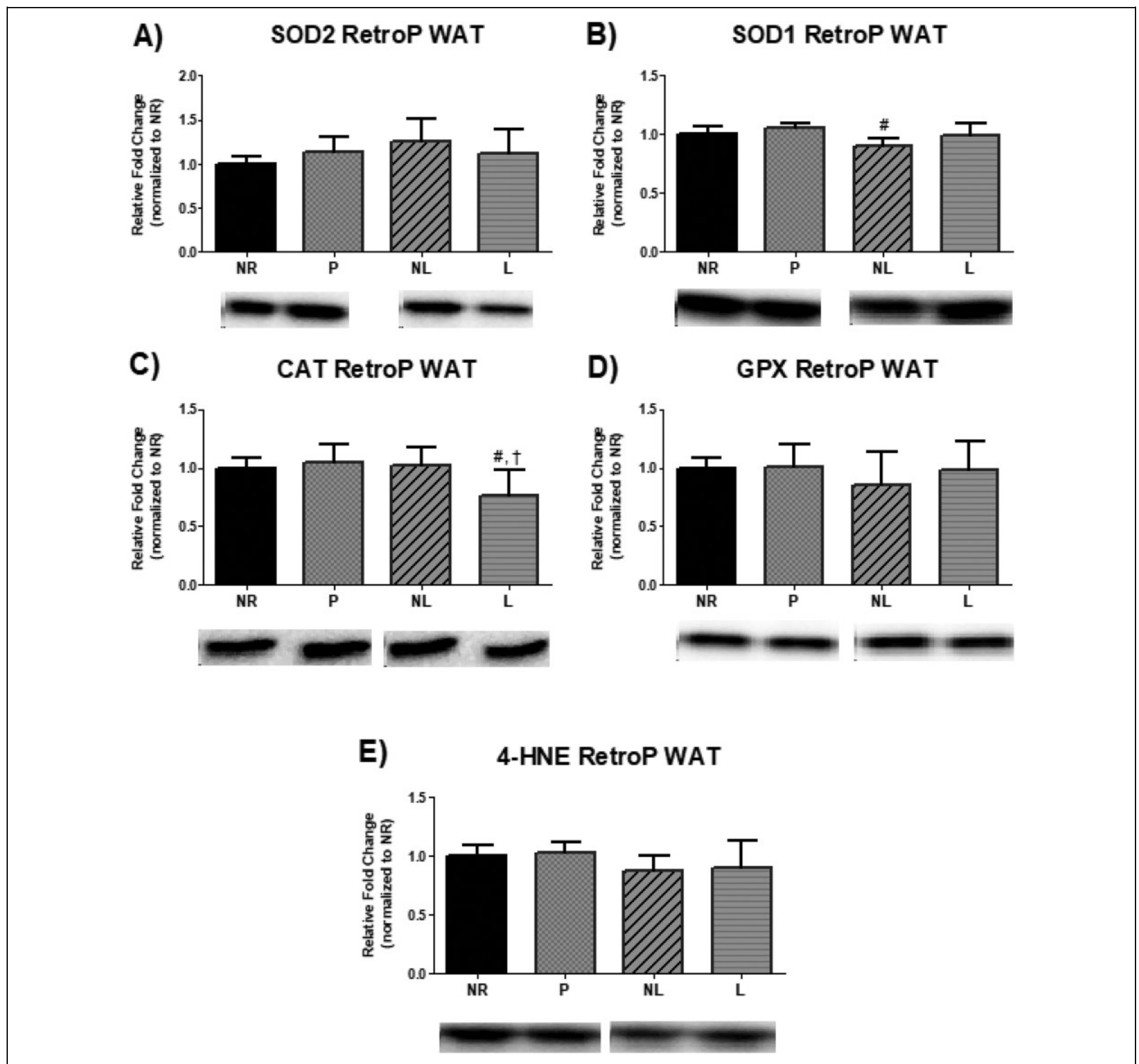

Figure 5. Markers of oxidative stress in retroperitneal (RetroP) white adipose tissue (WAT) for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants (A) SOD2, (B) SOD1, (C) CAT, and (D) GPX. In addition, data include (E) lipid peroxidation determined by 4-HNE, Representative blots are shown under the graphs. Data shown are mean ± standard deviation (SD). # Different from P ($P < .05$), and † different from NL ($P < .05$). SOD indicates superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

Markers of Metabolism are Differentially Regulated in Nonlactating and Lactating Animals

Following a dip in expression during pregnancy, liver PPARδ protein expression in nonlactating rats returned to levels comparable to NR rats, while lactating females displayed elevated PPARδ (Figure 2). The PPARδ is a member of a nuclear receptor superfamily that regulates transcriptional control of genes involved in glucose metabolism in the liver.[23,24] Thus, decreased liver PPARδ protein expression of pregnant animals would aid in sparing glucose for the fetus and reducing lipid oxidation, promoting lipid retention for lactation.[25]

Additionally, we report higher protein levels of PGC-1α in the liver of lactating animals compared to nonpregnant and pregnant animals (Figure 2). The PGC-1α is a well-known

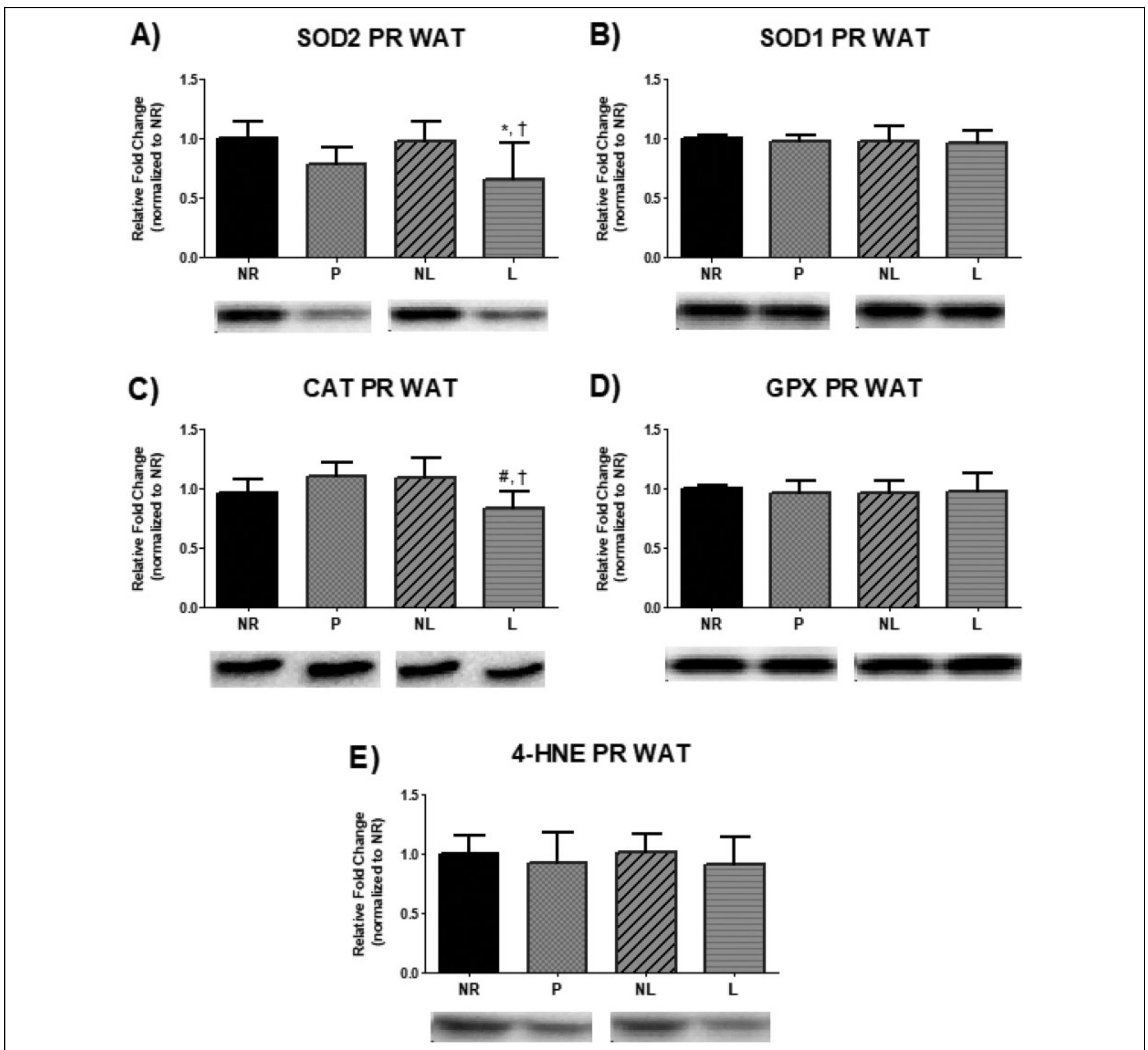

Figure 6. Markers of oxidative stress in perirenal (PR) white adipose tissue (WAT) for nonreproductive rats (NR), rats that were allowed to mate and became pregnant and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants (A) SOD2, (B) SOD1, (C) CAT, and (D) GPX. In addition, data include (E) lipid peroxidation determined by 4-HNE, Representative blots are shown under the graphs. Data shown are mean $\pm$ standard deviation (SD). *Different from NR (*P* < .05), # different from P (*P* < .05), and † different from NL (*P* < .05). SOD indicates superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

transcriptional regulator of genes involved in oxidative metabolism and mitochondrial biogenesis.[26] Thus, increased PGC-1α protein levels could serve as a means for driving mitochondrial biogenesis to support enhanced glucose metabolism facilitated by the increased PPARδ protein expression. Indeed, our results show that reproductive status yielded alterations in mitochondrial function between the groups (Table 1). The RCR, a metric used to determine mitochondrial function, was decreased when using both complex I and complex II substrates in liver mitochondria from pregnant animals. As this decrease in RCR appears to be driven by decreased active respiration (ie, state 3 respiration) in pregnant animals, this is perhaps another mechanism by which resources are spared for fetal consumption during pregnancy. In this regard, our findings of higher PGC-1α and PPARδ proteins during lactation may be indicative of the mechanisms involved in reversing pregnancy-induced mitochondrial alterations via the driving of transcriptional events involved in mitochondrial biogenesis

and oxidative metabolism. Conversely, nonlactating animals had higher liver mitochondrial complex activity at complex I compared to all groups (Table 3) without heightened levels of PGC-1α and PPARδ. Thus, the observed increased activity at complex I of nonlactating animals may be indicative of a separate undefined compensatory mechanism for reverting the suppressed mitochondrial function that occurs during pregnancy when the stimulus of lactation is not provided following birth. In skeletal muscle, PPARδ protein levels were decreased in nonlactating animals (Figure 2). This finding is important since PPARδ expression in skeletal muscle regulates genes involved in lipid oxidation,[23] and this could indicate a decrease in the capacity for lipid metabolism in skeletal muscle leading to increased adiposity.

Mitochondria Function and Oxidative Stress in Skeletal Muscle and WAT

In terms of skeletal muscle mitochondrial function, we observed that RCR was higher during lactation when using complex II substrate compared to pregnant animals (Table 2). This appears to be driven by a lower state 4 respiration. This finding agrees with previous work by our laboratory, in which RCR was increased in lactating mice in skeletal muscle being driven by a decrease in state 4 respiration.[27] State 4 can be a proxy for leak respiration, and thus, a reduction in leak may be useful for reducing metabolic cost of skeletal muscle. This finding may be further supported by previous findings of decreased UCP3 expression in skeletal muscle during lactation,[8,9] as increased UCP3 expression in skeletal muscle is associated with increased metabolism.[28]

ROS formation can occur during the processes of mitochondrial production of ATP leading to oxidative damage; however, endogenous antioxidant proteins within the cell exist in order to protect against oxidative damage.[11] An imbalance between antioxidant defense mechanisms and oxidant production is known as oxidative stress. In this regard, the free radical theory of aging has also been used to explain aging, in that oxidative damage across a lifetime causes disruption to cellular function causal in the processes of aging.[14] Due to the disposable soma theory and free radical theory of aging, researchers have posited that the increased energetic demands imposed by reproduction would result in elevated oxidant production, leading to cellular senescence and thus impacting longevity.[29-31] However, little empirical evidence exists on the physiological mechanisms that could support this claim. On the contrary, reports have demonstrated that markers of oxidative stress were unchanged or even decreased in liver during lactation compared to NR controls.[32-34] Our results show no differences in markers of lipid peroxidation and protein oxidation in liver or WAT (Figures 3, 5, and 6). Conversely, markers of lipid peroxidation were higher in the skeletal muscle of nonlactating animals compared to NR animals (Figure 4). This is an interesting finding, considering that according to the disposable soma theory nonlactating animals would be expending less energy toward milk synthesis and thus would be better able to allocate resources toward combating oxidative damage.

Finally, several markers of metabolism were measured in WAT (Figures 5 and 6). Converse to PPARδ's role in liver, PPARδ in white adipose tissue is involved in fat catabolism through oxidation of fatty acids.[35,36] PPARδ protein expression was significantly lower in females who did not lactate relative to NR and pregnant females. Given that the RetroWAT was significantly heavier in nonlactating rats, reduced levels of PPARδ likely played an important role for this effect. Furthermore, our findings of PPARδ being differentially regulated across 3 tissue types may further highlight the importance of PPARδ's involvement during reproduction but also that systemic events likely dictate a synchronized control system of its expression.

Conclusion

The goal of this study was to identify factors that play a role in increasing a female's probability of obesity and/or type II diabetes when she fails to sustain lactation. We report that that PPARδ in the skeletal muscle and RetroWAT of nonlactating rats was lower than in pregnant, lactating, and NR animals. This indicates that higher body fat and higher muscle mass were not associated with the retention of a physiological state that was set during pregnancy, as Stuebe and Rich-Edwards implied.[2] Instead, failure to lactate appears to contribute to a drop of PPARδ. While low expression of PPARδ has been linked to diabetes, high expression of this nuclear receptor is associated with cancer growth and proliferation.[37] If the observed effects are sustained, low expression of PPARδ may play a greater role in the development of obesity and type II diabetes. Future studies should evaluate variation in PPARδ protein expression in the weeks and months after reproduction has ended to determine whether this effect is sustained. The results of this study indicate that targeting PPARδ with therapeutic drugs shortly after women make the decision to not breast-feed could prove valuable in rescuing women from subsequent obesity and diabetes.

Declaration of Conflicting Interests

The author(s) declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by US National Institutes of Health grant R03 HD083654.

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