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Neonatal nicotine exposure affects adult rat hepatic pathways involved in endoplasmic reticulum stress and macroautophagy in a sex-dependent manner

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Original Article

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Abstract

Nonalcoholic fatty liver disease (NAFLD) involves changes in hepatic pathways, as lipogenesis, oxidative stress, endoplasmic reticulum (ER) stress, and macroautophagy. Maternal nicotine exposure exclusively during lactation leads to fatty liver (steatosis) only in the adult male offspring, not in females. Therefore, our hypothesis is that neonatal exposure to nicotine sex-dependently affects the signaling pathways involved in hepatic homeostasis of the offspring, explaining the hepatic lipid accumulation phenotype only in males. For this, between postnatal days 2 and 16, Wistar rat dams were implanted with osmotic minipumps, which released nicotine (NIC; 6 mg/Kg/day) or vehicle. The livers of offspring were evaluated at postnatal day 180. Only the male offspring that had been exposed to nicotine neonatally showed increased protein expression of markers of unfolded protein response (UPR), highlighting the presence of ER stress, as well as disruption of the activation of the macroautophagy repair pathway. These animals also had increased expression of diacylglycerol O-acyltransferase 1 and 4-hydroxynonenal, suggesting increased triglyceride esterification and oxidative stress. These parameters were not altered in the female offspring that had been neonatally exposed to nicotine, however they exhibited increased phospho adenosine monophosphate-activated protein kinase pAMPK expression, possibly as a protective mechanism. Thus, the disturbance in the hepatic homeostasis by UPR, macroautophagy, and oxidative stress modifications seem to be the molecular mechanisms underlying the liver steatosis in the adult male offspring of the nicotine-programming model. This highlights the importance of maternal smoking cessation during breastfeeding to decrease the risk of NAFLD development, especially in males.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and has increased prevalence,[1] especially among men.[2] Its global prevalence is 25.24%, with 33%–38% in men and 15.8%–23% in women.[2,3] The prevalence of hepatic steatosis, a metabolic disturbance associated with NAFLD, is also higher in men than in premenopausal women (19% *vs.* 9%), although it was similar between men and postmenopausal women (19% *vs.* 22%), reinforcing the protective role of estrogen.[4] NAFLD is a multifactorial disease with alterations in many different cellular processes, such as increased de novo lipogenesis, impaired β-oxidation, impaired VLDL secretion, insulin resistance, oxidative stress,[5] mitochondrial dysfunction, endoplasmic reticulum (ER) stress,[6] and macroautophagy.[7] These alterations can act in synergistic and complex ways in the liver and NAFLD development and progression feature a multiple-hit hypothesis.[8] This is the most accepted theory to explain the intensity of NAFLD, especially in individuals with epigenetic predispositions, which are adaptive changes in these cellular processes.[5,9]

The maternal environment during pregnancy and/or lactation affects susceptibility to metabolic diseases, including NAFLD, which supports the theory of the developmental origins of health and disease (DOHaD).[10] In this context, it has been established that unhealthy maternal diets, such as high-fat or high-sugar diets,[11,12] promote higher offspring susceptibility to NAFLD. In the same context, maternal exposure to environmental pollution or drugs, such as cigarette smoke, impacts the liver homeostasis of offspring in animal models[13,14] and increases the risk of NAFLD in human progeny.[15]

Cigarettes are used by approximately 20% of pregnant German and American women[16,17] and by an even higher percentage of women after delivery,[18] exposing the child to nicotine via

smoke and through breast milk.[19] Globally, 52.9% of smoking women continue to smoke daily during pregnancy and breastfeeding,[20] and the prevalence of tobacco use among breastfeeding women ranges from 3.61% to 5.2%.[21,22] Approximately 51.8% of women who smoked during the perinatal period are light smokers, smoking up to 10 cigarettes per day. However, approximately 13.5% are heavy smokers, smoking ≥ 20 cigarettes per day.[20] Additionally, nicotine replacement therapy could be recommended during this perinatal period to reduce exposure to tobacco smoke, although perinatal nicotine exposure would be sustained.[23] These data are worrisome since nicotine diffuses passively and quickly through the mammary ducts; in fact, nicotine in milk accounts for 85% of maternal serum nicotine.[24]

Our group has shown the negative impact of maternal nicotine exposure during the lactation period in a rodent model,[25] which promotes increased body mass and visceral adiposity in male adult offspring[26] without affecting female adult offspring.[27] Interestingly, liver homeostasis was also affected in a sex-dependent manner. Male offspring exposed to nicotine early in adulthood exhibited increased liver markers of oxidative stress, de novo lipogenesis, and increased hepatic triglyceride (TG) levels (steatosis) in the presence of normal plasma lipids.[28,29] In early exposed females, despite normal hepatic morphology and TG content, these subjects showed paradoxically higher protein expression of ACC-1 and FAS (acetyl-CoA carboxylase-1 and fatty acid synthase, respectively) in the liver, as well as higher cholesterol and TG in plasma.[29]

It is well established that the liver plays a pivotal role in controlling lipid metabolism and that changes in the pathways involved in hepatic homeostasis, such as ER stress and autophagy, could contribute to the sexual dimorphism phenotype observed in this model. Increased hepatic lipogenesis can induce oxidative and ER stress based on offspring susceptibility.[30,31] Similarly, chronic ER stress, which is a result of intense activation of the unfolded protein response (UPR), can increase de novo lipogenesis and disturb VLDL assembly, favoring hepatic steatosis.[6] In addition, the impairment of autophagy is a common finding in many NAFLD models,[32,33] which damages lipophagy and mitophagy and disturbs liver homeostasis.[33] Autophagy is a conserved cellular process in which cytoplasmic contents, such as damaged organelles, are degraded by lysosomes.[34,35] Macroautophagy is the most prevalent form, which involves a double-membrane sequestering compartment that matures into an autophagosome. This structure fuses with the lysosome, causing the degradation of its contents and resulting in recycling of macromolecules that are released into the cytosol.[35]

\A chronic response and the inefficient activation of repair pathways, such as the UPR and macroautophagy, could cause hepatic injury and impair metabolic homeostasis. In the present study, we hypothesized that neonatal nicotine exposure increases the risk of NAFLD in male progeny through sex-dependent activation of pathways involved in liver injury progression, which affects outcome intensity and explains the sex-related differences observed in this programming model. We evaluated liver markers of ER stress, such as PERK (protein kinase R (PKR)-like ER kinase), eIF2 α (eukaryotic translational initiation factor 2 α), sXBP-1 (spliced X-box binding protein-1) and CHOP (C/EBP homologous transcription factor), and markers of macroautophagy, such as LC3 (microtubule-associated protein B-light chain 3) and p62, in the adult male and female offspring of dams that were exposed to nicotine during the breastfeeding period.

Material and methods

Experimental design

The experimental procedures were approved by the Ethics Committee on Animal Care of the Biology Institute of the State University of Rio de Janeiro (CEUA/015/2017) in accordance with Brazilian Law no. 25 11.794/2008.[36] The experimental design followed the ARRIVE guidelines.[37] The animals were kept in a room with controlled temperature (23 ± 2 °C) and light/dark cycles (lights on 7 am and lights off 7 pm). The animals had free access to water and chow (Nuvilab, Sogorb, São Paulo, SP, Brazil).

Fourteen pregnant female Wistar rats were housed in individual cages (10–12 weeks of age). At birth, which was postnatal day 0 (PND0), the litters were adjusted to six pups per dam (three males and three females) to improve breastfeeding performance. At PND2, lactating dams were randomly divided into two groups (saline or nicotine) and were anesthetized with thiopental (30 mg/kg) for the subcutaneous implantation of osmotic minipumps on the back. The osmotic minipumps released a saline solution (control group, C, $n = 7$) or a nicotine-free base diluted in saline (Alzet, Cupertino, CA, USA) (nicotine group, NIC, $n = 7$), delivering a dose of 6 mg/kg/day of nicotine from PND2 until PND16. This dose is equivalent to that in heavy smokers (≥20 cigarettes per day).[26,27] Approximately 52.9% of smoking women continue to smoke daily during the perinatal period,[20] and 13.5% are considered heavy smokers.[20] Cotinine, a marker of nicotine exposure, was detected in the dams' milk and serum, which were similar to serum levels of heavy smokers,[38,39] and in the pups' serum.[40] The cotinine concentrations in dam milk and serum were 225.8 ± 7.1 ng/ml and 239.7 ± 25.2 ng/ml, respectively. However, the offspring exhibited a serum cotinine concentration of 20 ng/ml.[40] From PND21 (weaning) onward, the animals were kept in groups of 3–4 per cage (same sex) until euthanasia (1 pup/sex/litter). The remaining rats were assigned to other studies. From weaning until adulthood, the animals had free access to water and standard rodent chow (Nuvilab, Sogorb, São Paulo, SP, Brazil). The estrous cycles of female offspring were monitored daily in the last month of life using a vaginal swab technique. The offspring were killed by decapitation at PND180, and all females were in the diestrus phase to reduce the interference of hormonal variations. The liver was dissected, snap frozen in liquid nitrogen and stored at −80°C for further analyses.

Western blotting analysis

In the present study, we examined liver samples from a previously published model.[41] Liver samples (30 mg) were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; SDS 0.1%; 5 mM EDTA; 50 mM NaF; 30 mM Na4P2O7; 1 mM Na3VO4) containing a protease inhibitor cocktail (La Roche Ltd., SW). After centrifugation (7,500 ×g/15 min/4°C), the total protein content of the supernatant was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA), and 20 µg samples were diluted in Laemmli buffer. The samples were heated to 95°C for 3 minutes and immediately cooled on ice, as previous recommended.[42] After, an electrophoresis in an SDS polyacrylamide gel with a molecular weight standard (Kaleidoscope, Bio-Rad™, USA) was performed. More details on different gel concentrations and biomarkers used can be found in the Supplementary Material. The samples were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, UK) in a Semi-dry system (15 V for 40 minutes) (Trans-Blot® Turbo™ Transfer System,

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Table 1. Primary antibodies used in western blotting analysis

Protein	Primary antibody	Secondary antibody
Lipid metabolism		
Carnitine Palmitoyl transferase 1a (CPT1a)	Abcam (#ab128568) 1:800 dilution	Sigma-Aldrich (#B8520) 1:10000 dilution
Diacylglycerol O-acyltransferase 1 (DGAT1)	Abcam (#ab122924) 1:1000 dilution	Sigma-Aldrich (#B7024) 1:10000 dilution
Oxidative Stress		
4-Hydroxynonenal (4-HNE)	Abcam (ab46545) 1:1000 dilution	Invitrogen (#656120) 1:2000 dilution
UPR		
Phospho-PKR-like ER kinase (p-PERK)	Santa Cruz Biotechnology (sc-32577) 1:400 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
PKR-like ER kinase (PERK)	Santa Cruz Biotechnology (sc-9477) 1:200 dilution	Sigma-Aldrich (#B7024) 1:10000 dilution
Phospho-eukaryotic translational initiation factor 2 α (peIF2 α)	Cell signaling (#3398) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
Eukaryotic translational initiation factor 2 α (eIF2 α)	Cell signaling (#2103) 1:1000 dilution	Sigma-Aldrich (#B8520) 1:10000 dilution
Spliced X-box binding protein-1 (sXBP-1)	Cell signaling (#12782) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
C/EBP homologous transcription factor (CHOP)	Cell signaling (#2895) 1:1000	Sigma-Aldrich (#B8520) 1:10000 dilution
Autophagy		
SQSTM1/p62 (p62)	Cell signaling (#5114) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
Microtubule-associated protein B-light chain 3 (LC3)	Cell signaling (#2775) 1:600 dilution	Sigma-Aldrich (#B7389) 1:5000 dilution
Others		
Sirtuin 1 (SIRT1)	Cell signaling (#9475) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
Caspase 3 (CASP-3)	Invitrogen (#437800) 1:800 dilution	Sigma-Aldrich (#B8520) 1:10000 dilution
AMP-activated protein kinase (pAMPK) *	Cell signaling (#2532S) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
Phosphorylated AMP-activated protein kinase (pAMPK) *	Cell signaling (#25355) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
Loading control		
GAPDH	Cell signaling (#5174) 1:1000 dilution	Sigma-Aldrich (#B7389) 1:10000 dilution
β-actin	Sigma-Aldrich (A2228) 1:4500 dilution	Sigma-Aldrich (#B8520) 1:10000 dilution

This antibody detects the alpha subunit of AMPK.

Bio-Rad™ USA). The membranes were incubated with Tris-buffered saline (TBS) with 5% BSA to block nonspecific binding sites and then fragments were incubated with specific primary antibodies (overnight) against proteins involved in lipid metabolism, oxidative stress, the UPR and macroautophagy (Table 1). These primary antibodies were diluted in TBS with BSA 2%. Next, membranes were incubated with specific secondary antibodies (90 min), followed by streptavidin incubation (60 min). Antigen visualization was carried out by chemiluminescent reagents (Bio-Rad™, USA) and the ImageQuant Las 500 system (GE Healthcare, UK). Band intensities were quantified by optical densitometry using ImageJ software (Media Cybernetics, MD, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or beta-actin (β-actin) was used as the internal control, and the results are expressed relative to the control group. We performed a stripping protocol on the membranes in which we marked the total and phosphorylated protein as well as 4-HNE and eIF2 α using a solution of glycine (0.2 M), SDS (0.1%) and Tween 20 (1%) (pH 2.2). Prior to the next incubation, we validated the stripping process by verifying the absence of a signal using chemiluminescent reagents and the ImageQuant Las 500 system.

Statistical analysis

GraphPad Prism 6.0 software (GraphPad Software Inc., CA, USA) was used to prepare the graphics and perform the statistical analysis.

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The Kolmogorov–Smirnov one-sample test (K-S) was used to assess the normality of the distributions of each of the variables. The results are shown as the mean ± SEM. The NIC group was compared to the C group of the same sex, since for Western blot analysis, male and female samples were evaluated on different gels. The data were analyzed by unpaired Student's *t*-test (two-tailed), and differences were considered significant at $p < 0.05$. To qualify the magnitude of the differences obtained by Student's *t*-test, the effect size was calculated. Effect size data are provided as Eta-squared (η^2: small > 0.1, moderate > 0.3, large > 0.5).

Results

Briefly, as described before,[41] body weight was higher in NIC males (+14.4%) than in the control group, and there were no differences in female offspring, which is consistent with our previous studies.[26,29] Only NIC males exhibited higher adiposity and hepatic lipid deposition, with 1.39-fold higher hepatic TGs, despite no changes in serum TGs, as already reported.[29] NIC females only showed higher serum TGs.[29] We used the samples of this previously published model[41] and investigated the molecular mechanism of this sexual dimorphism in the liver. As shown in Fig. 1a, NIC male offspring showed no changes in PERK phosphorylation or total content in the liver. However, these animals showed increased levels of the downstream target phosphorylated eukaryotic translational initiation factor 2 α (peIF2 α) (+62% *vs.* C males, $p = 0.043$, $\eta 2 = 0.30$) (Fig. 1a). NIC males showed no changes in total peIF2 α, which increased the peIF2-α/eIF2 α ratio (+86% *vs.* C males, $p = 0.002$, $\eta^2 = 0.56$) (Fig. 1a). Additionally, these animals exhibited higher CHOP levels in the liver (+70% *vs.* C males, $p = 0.030$, $\eta^2 = 0.35$) but no changes in sXBP-1 levels (Fig. 1a). Interestingly, NIC female offspring showed reduced total eIF2 α levels in the liver (-40% *vs.* C females, $p = 0.003$, $\eta 2 = 0.56$) but not changes in phosphorylated eIF2-alpha levels, which increased the peIF2-α/eIF2 α ratio (+127% *vs.* C females, $p<0.001$, $\eta^2 = 0.77$). NIC female offspring also exhibited no changes in PERK and CHOP levels in the liver (Fig. 1b).

The NIC male offspring exhibited higher hepatic carnitine palmitoyl transferase 1a (CPT1a) (+77% *vs* C males, $p = 0.024$, $\eta^2 = 0.38$), higher hepatic 4-hydroxynonenal (4-HNE) (+84% *vs* C males, $p = 0.002$, $\eta^2 = 0.68$) and higher diacylglycerol O-acyltransferase 1 (DGAT1) levels (+117% vs C males, $p = 0.020$, $\eta 2 = 0.43$) than their respective controls (Fig. 1c). Interestingly, NIC female offspring showed no changes in CPT1a, 4-HNE or DGAT1 levels compared to their respective controls (Fig. 1d).

We also evaluated the macroautophagy pathway. In NIC male offspring, we observed an increase in LC3-II levels (+79% *vs.* C males, $p = 0.009$, $\eta 2 = 0.59$, Fig. 2a) and the LC3-II/LC3-I ratio (+128% *vs.* C males, $p = 0.03$, $\eta^2 = 0.41$, Fig. 2a), and there were elevated p62 levels (+88% *vs.* C males, $p = 0.015$, $\eta^2 = 0.42$, Fig. 2a). On the other hand, NIC female offspring showed no changes in LC3-I, LC3-II, the LC3-II/LC3-I ratio or p62 levels (Fig. 2b).

Although SIRT1, pAMPK and total AMPK expression, and the pAMPK/AMPK ratio were unchanged, NIC male offspring showed higher caspase 3 (CASP-3) expression (238+% *vs.* C males, $p = 0.05$, $\eta^2 = 0.31$, Fig. 2c). On the other hand, NIC female offspring exhibited a marked increase in the levels of pAMPK (+172% *vs.* C males, $p = 0.05$, $\eta^2 = 0.33$, Fig. 2d), total AMPK (+107% *vs.* C males, $p = 0.022$, $\eta^2 = 0.36$, Fig. 2d), and the pAMPK/AMPK ratio (+170% *vs.* C males, $p = 0.039$, $\eta^2 = 0.31$, Fig. 2d) compared to control offspring that showed no changes in SIRT 1 or CASP-3 levels.

Discussion

Our previous study showed that postnatal nicotine exposure affected hepatic morphology in adult male and female offspring in dissimilar ways, promoting hepatic steatosis only in NIC male offspring.[29] In adulthood, only NIC male offspring exhibited higher body weight, adiposity, and hepatic lipid accumulation, as previously described.[29] Here, we suggest that these sex-related differences in phenotype are the result of modifications in the pathways involved in maintaining liver homeostasis and might contribute to hepatic lipid accumulation only in male offspring.

Hepatic lipid accumulation could activate the UPR pathway, which is involved in repair. Conversely, intense UPR activation could increase lipogenesis, promoting hepatic lipid accumulation.[43] Here, we evaluated biomarkers associated with this pathway and observed sex-dependent changes in NIC offspring. Higher peIF2 α levels occurred only in NIC male offspring, even in the absence of changes in PERK phosphorylation, suggesting the contribution of other kinases in the stress response.[44] This increase in eIF2 α phosphorylation is required for the UPR pathway and for hepatic TG accumulation in response to a high-fat diet,[45] suggesting its contribution to the steatosis observed in NIC male offspring. Interestingly, NIC female offspring showed only reduced total eIF2 α levels. Therefore, it seems that although there is an increase in the peIF2-α/eIF2 α ratio in NIC animals of both sexes compared to controls, UPR activation seems to occur only in NIC males. Although there was lower total eIF2-alpha expression in NIC females, they had normal activation of protein (peIF2-alpha levels), suggesting integrity of its role. However, it is possible that NIC females have a higher risk of failure when subjected to a second insult manner.

The UPR is a repair pathway responsible for adaptation to cellular stress, but if the cell is unable to restore homeostasis, then ER stress-related apoptosis is triggered. This apoptosis pathway involves CHOP activation,[46] which was selectively higher in NIC male offspring, which was consistent with the robust increase in CASP-3 expression in these animals. This protease is part of the apoptotic signaling cascade, which is activated in hepatic disease and linked to disease severity.[47]

An increased predisposition to ER stress in male rats compared to female rats was previously described, and it seems to be the result of testosterone-mediated activation of the UPR pathway in the liver.[30] Although NIC males in the same programming model show lower testosterone levels, androgen receptor levels in thyroid tissue are upregulated.[25] Therefore, in the liver, it is possible that the androgen receptor is positively regulated, normalizing or even increasing the effects of testosterone on this tissue. Conversely, if testosterone levels indicate lower action in the liver in NIC males, it can contribute to hepatic lipid accumulation, since orchidectomized rats exposed to a high cholesterol diet exhibit higher hepatic lipid levels.[48] Since estrogen has a protective role in hepatic ER stress development,[49] this could help to explain the improved liver profiles of NIC females.

This hepatic UPR activation in NIC male offspring could contribute to the steatosis profile described previously,[29] since it increases the transcription of lipogenic genes, such as SREBP-1[43] and lipogenic enzymes, as recently reported in these animals.[29]

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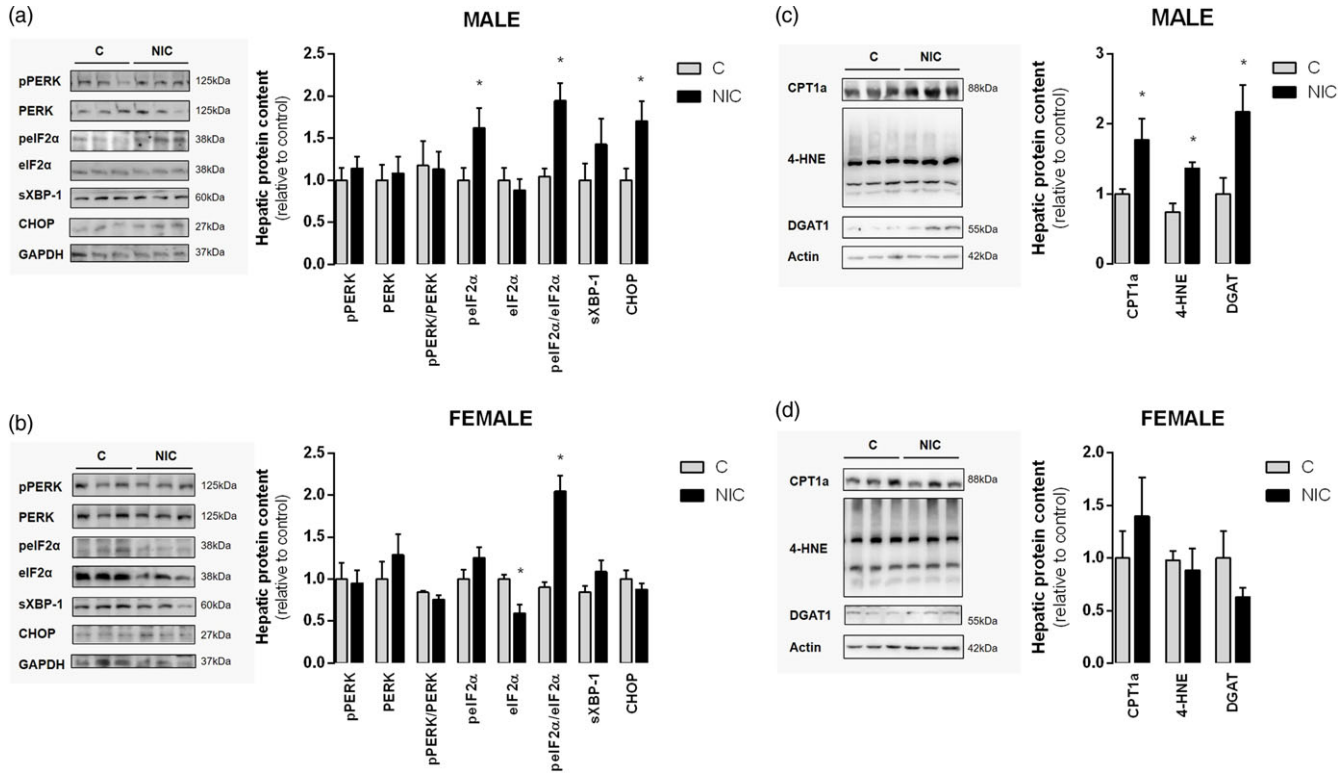

Figure 1. Markers of the unfolded protein response (UPR) pathway and the expression of CPT1a, 4-HNE, and DGAT1 in the liver of adult offspring (PND180) of both sexes. (***a***) Representative blots and relative expression of markers of the UPR pathway in male offspring. (***b***) Representative blots and relative expression of markers of the UPR pathway in female offspring. (***c***) Representative blots and relative expression of CPT1a, 4-HNE, and DGAT1 in male offspring. (***d***) Representative blots and relative expression of CPT1a, 4-HNE, and DGAT1 in female offspring. Abbreviations: phospho-PKR-like ER kinase (pPERK); PKR-like ER kinase (PERK); phospho-eukaryotic translational initiation factor 2 α (peIF2 α); eukaryotic translational initiation factor 2 α (eIF2 α); spliced X-box binding protein-1 (sXBP-1); C/EBP homologous transcription factor (CHOP); carnitine palmitoyl transferase 1a (CPT1a); 4-hydroxynonenal (4-HNE); and diacylglycerol O-acyltransferase 1 (DGAT1). Data are expressed as the mean ± SEM; $n = 6$-7 rats from different litters per group (1 pup/sex/litter); representative blot from 3 animals. Statistical analysis was performed using unpaired Student's *t*-test. $^{*}p<0.05$.

Additionally, the higher DGAT1 levels in male NIC offspring could contribute to hepatic TG accumulation by increasing the acylation of diacylglycerol to form triacylglycerol.[50] However, we cannot disregard the additional contribution of DGAT2, since this isoform has been involved in the incorporation of fatty acids from cellular de novo lipogenesis.[51]

In addition, in NIC males, there is a compensatory increase in β-oxidation, as indicated by higher CPT1a levels, which can increase the generation of reactive oxygen species (ROS).[5] Reinforcing our previous observation,[28] only NIC male offspring exhibited higher 4-HNE expression, which is a marker of lipid oxidative damage and might contribute to UPR activation.

Another pathway involved in the maintenance of homeostasis that is activated by ER and oxidative stress is macroautophagy.[52] This pathway reduces cellular stress by recycling organelles and substrates[7,53] through phagosome formation. This structure is responsible for sequestration of the contents to be degraded,[7] and microtubule-associated protein B-light chain 3 (LC3-I) conversion to LC3II is a limiting step.[54] The macroautophagy process is only complete when this structure is fused with a lysosome, which is responsible for the degradation of the internal contents, and this process is mediated by autophagic receptors, such as the ubiquitin-binding protein p62. This protein is continuously produced and degraded, which reflects autophagic flux.[53] Here, the LC3-II/LC3-I ratio and p62 regulation in NIC males suggest disruption of the macroautophagy pathway, compromising the degradation of damaged organelles and substrates, such as lipids,[53] thereby contributing to TG accumulation in the liver. Interestingly, these changes were not present in the female offspring in the NIC group.

The analysis of the LC3-II/LC3-I ratio has limitations due to differential expression and affinities for antibodies, and we performed LC3-II normalization to a loading control to improve data interpretation.[55] We observed the same profile, reaffirming the interpretation of the findings. However, it is important to consider the limitations of evaluating protein expression without the use of lysosomal fusion or protease inhibitors when interpreting autophagic flux.[54]

Since the UPR and autophagy are repair pathways, their adaptive changes could contribute to metabolic disorders in offspring, such as in the offspring in a maternal undernutrition model.[56] A maternal high-fat diet affects the UPR pathway in the metabolic tissues of offspring, such as the pancreas,[57] liver,[58,59] and hypothalamus,[60] increasing susceptibility to diabetes, NAFLD, hyperphagia, and obesity.

The impairment of the macroautophagy pathway and the intense UPR activation in NIC males might favor apoptosis, as demonstrated by the increase in hepatic CHOP expression and CASP-3 expression. Additionally, the absence of these changes in NIC females may be associated with the absence of liver steatosis. Although SIRT1 expression showed no changes in among experimental groups, we observed a marked increase in the levels of pAMPK only in NIC female offspring. This kinase plays a central role in cellular metabolism regulation, and its pharmacological activation has been implicated in improvements in liver

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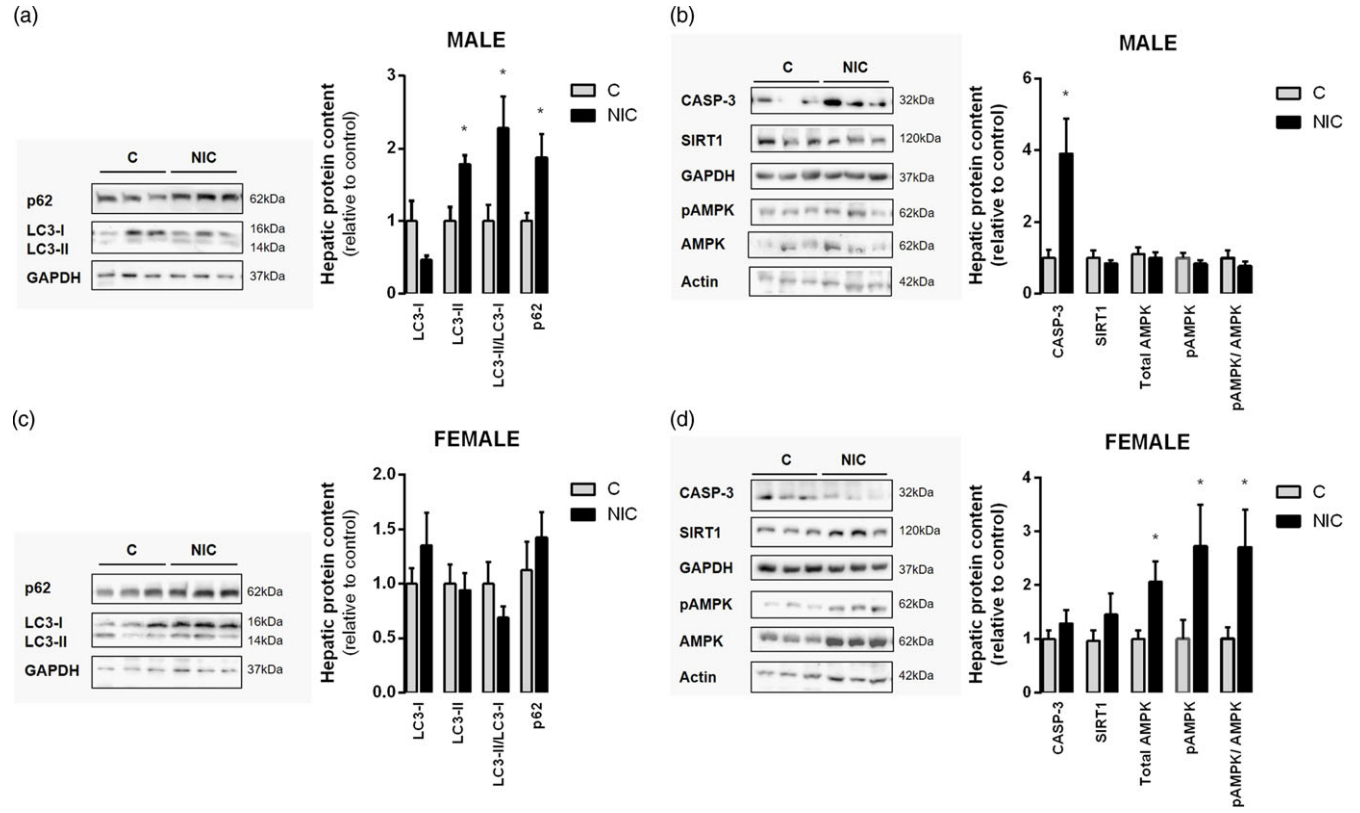

Figure 2. Markers of the macroautophagy pathway and expression of CASP-3, SIRT1, and pAMPK in the livers of adult offspring (PND180) of both sexes. (***a***) Representative blots and relative expression of markers of the macroautophagy pathway in male offspring. (***b***) Representative blots and relative expression of markers of the macroautophagy pathway in female offspring. (***c***) Representative blots and relative expression of CASP-3, SIRT1, total AMPK, pAMPK, and pAMPK/total AMPK ratio in male offspring. (***d***) Representative blots and relative expression of CASP-3, SIRT1, total AMPK, and pAMPK in female offspring; microtubule-associated protein B-light chain 3 (LC3) (LC3-II/LC3-I ratio); SQSTM1/p62 (p62); caspase 3 (CASP-3); sirtuin 1 (SIRT1); phosphorylated AMPK (pAMPK). Data are expressed as the mean ± SEM; $n = 6$-7 rats from different litters per group (1 pup/sex/litter); representative blot from 3 animals. Statistical analysis was performed using unpaired Student's *t*-tests. *$p<0.05$.

injury.[61] The mechanism of this increase may involve liver protection in NIC female offspring to prevent NAFLD development.[61–64] AMPK activation can attenuate ER stress,[63] suppress de novo lipogenesis by regulating the activity of key enzymes and increase fatty acid oxidation.[61] These AMPK effects conceivably counteract the liver damage of early nicotine exposure. However, the long-term effects of AMPK activation must be investigated in our model.

Nicotine is an important component of cigarette smoke that disrupts perinatal development.[23] Interestingly, our group has shown that differences in liver metabolic outcomes in adulthood exist between exposure to tobacco smoke and exposure only to nicotine during the lactation period.[29,65] Male and female offspring from dams exposed to tobacco smoke show increased hepatic lipogenesis markers but an absence of lipid accumulation in this tissue.[65] Although serum cotinine levels were approximately 7-fold higher in the pups of the tobacco smoke model than in the pups in the nicotine model,[25] male nicotine-exposed offspring showed higher hepatic lipogenesis and steatosis.[29]

These data suggest a strong impact of nicotine on lipid metabolism in the liver, even though other tobacco smoke compounds may delay the development of steatosis. Indeed, nicotine is able to increase obesity-induced hepatic steatosis,[66] probably through its ability to induce both oxidative and ER stress.[67] On the other hand, a beneficial effect of nicotine on the serum lipid profile, hepatic steatosis, and ER stress in the livers of diet-induced obese adult rats has also been reported.[68] In our model, nicotine exposure was restricted to the lactation period, discarding the possibility that nicotine directly affected these pathways in adulthood but suggesting the involvement of epigenetic and adaptive changes, which are influenced by the sex of the offspring. Indeed, sexual dimorphism was described previously in the nicotine model;[27,29] female offspring were less affected than male offspring.

The absence of hepatic changes in the signaling pathways in NIC female offspring seems to be involved in the hepatic protection observed in this sex. In a previous study,[29] despite the unchanged cytoarchitecture and signaling pathways involved in liver homeostasis, NIC females had slightly disordered lipid metabolism. One limitation of the current study is the lack of a description of the mechanism responsible for the higher plasma lipids observed in NIC females in this previously published model.[29] In response to the increased ACC-1 and FAS in the liver in female offspring, it is possible that increased VLDL synthesis and assembly is present, increasing plasma lipids but preserving liver homeostasis and morphology.[29] The impact of this mechanism over time remains to be determined, however. A time-dependent effect seems to occur in NIC males. Previously, at PND120, we demonstrated that NIC males showed lower plasma TG levels and steatosis degree 1, as well as higher ACC-1 and FAS expression compared to control offspring.[29] At the same age, NIC females had normal plasma TG levels and preserved hepatocyte architecture, and there were changes in lipogenic enzyme expression.[29] Later, at PND180, NIC males progressed to show

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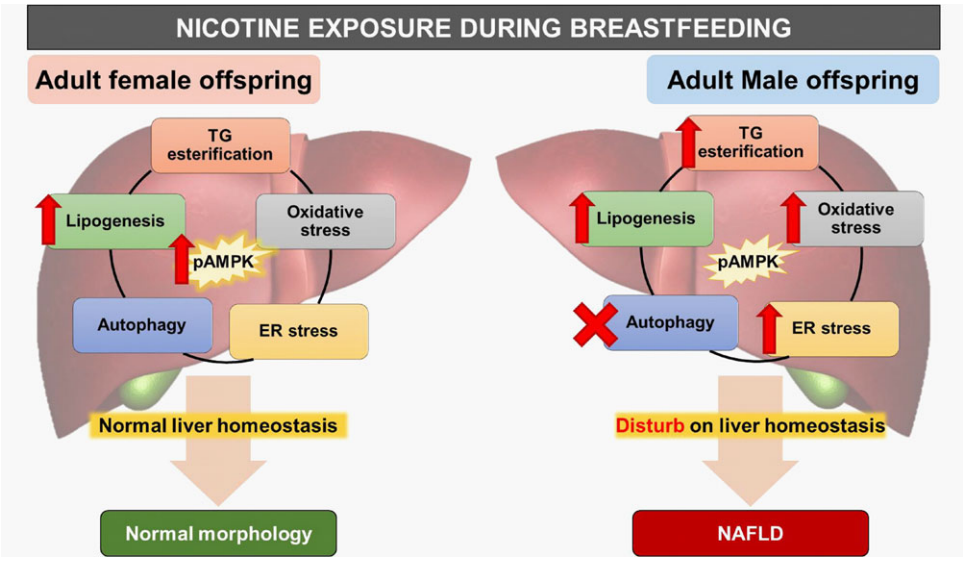

Figure 3. Sexual dimorphism in the signaling pathways involved in nonalcoholic fatty liver disease (NAFLD) development in adult rats exposed to nicotine during breastfeeding. In rats, maternal nicotine (NIC) exposure during the lactation period differentially affects liver homeostasis in adult progeny. NIC males show increased lipogenesis markers, triglyceride (TG) esterification, oxidative stress, and endoplasmic reticulum (ER) stress and disruptions in the macroautophagy repair pathway, highlighting a hepatic disturbance, which could justify the NAFLD phenotype. Conversely, NIC females show only mildly increased lipogenesis and increased pAMPK levels, which may play a protective role, preserving hepatic homeostasis and morphology.

higher hepatic TG levels and steatosis degrees 1 and 2, while NIC females exhibited higher plasma TG and CHOL levels, no changes in hepatic morphology and TG levels, and higher total ACC-1, FAS and pAMPK expression.29 Therefore, the precocious activation of hepatic lipogenesis in NIC male offspring compared to females of the same age[29] seems to be responsible for the alterations in hepatic homeostasis, which could also occur later in life in NIC females or under metabolic stress challenge.

We cannot discard the contribution of the peripheral changes in lipid metabolism. For example, only NIC males were shown to have hyperinsulinemia, hypothyroidism, lower hepatic expression of thyroid hormone targets, and hyperleptinemia,[26,69] which could directly affect lipid metabolism. Moreover, at PND180, only NIC male offspring exhibited increased body mass and adiposity compared to control offspring.[70]

Thus, our study provides evidence that the steatosis in NIC males[29] involves increased TG esterification, ER, and oxidative stress associated with the disruption of macroautophagy flux, and disturbances in liver homeostasis and the control lipid metabolism. The involvement of these pathways in steatosis susceptibility has been previously described in a programming model of maternal obesity.[31,71] Additionally, it is conceivable that AMPK activation in NIC female offspring is involved in the protection against NAFLD and maintenance of liver homeostasis (Fig. 3). For the first time, we describe this involvement in offspring programmed by nicotine exposure during the lactation period. In addition, we highlight the role of the activation of these pathways in the sex-dependent differences in the lipid metabolism of offspring.

Conclusion

Nicotine exposure during the lactation period negatively affects signaling pathways involved in liver homeostasis only in adult male offspring. The findings described here may be responsible for cellular stress, which helps to explain the increased susceptibility to NAFLD in males, particularly when compared to liver protection observed in female offspring. The current observations shed light on the molecular mechanisms involved in the programming of liver steatosis triggered by early nicotine exposure, highlighting the importance of smoking cessation, not only during pregnancy but also during the critical period of breastfeeding. This knowledge may help in the development and implementation of therapeutic strategies to mitigate the metabolic impact of maternal perinatal exposure to nicotine.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174423000326.

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Author contribution. *LLS and PCL* were responsible by the conceptualization; *TCP* performed the animal experimental model and the acquisition of samples; *LLS, CLR, and ACM* were responsible by the data acquisition, analysis and interpretation; *EGM and PCL* were responsible by the funding acquisition; *LLS* was responsible by the prepare of original draft; *LLS, ACM, EGM, and PCL* performed the revision of manuscript.

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Competing interests. None.

Ethical standards. The authors report that all procedures performed this work comply with the ethical standards of relevant national guides on the care and use of laboratory animals in accordance with the Brazilian Law no.25 11.794/2008 and have been approved by the committee on Animal Care of the Biology Institute of the State University of Rio de Janeiro (CEUA/015/2017).

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