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Glutathione and Inter-α-trypsin inhibitor heavy chain 3 (Itih3) mRNA levels in nicotine-treated Cd44 knockout mice

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Chemical compounds studied in this article:
L-Nicotine (89594)
L-Glutathione reduced, also GSH (124886)
L-Glutathione oxidized or oxiglutatione, also GSSG (65359)
β-Nicotinamide adenine dinucleotide 2′-phosphate or NADPH (16218775)
Dimethyl sulfoxide or DMSO (679)
5-Sulfosalicylic acid dihydrate (2723734)
5,5′-Dithiobis(2-nitrobenzoic acid) or DTNB (6254)
4-Vinylpyridine (7502)

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Gender

ABSTRACT

Cluster of differentiation 44 (Cd44), a hyaluronan receptor, and the secreted hyaluronan-binding protein Inter-α-trypsin Inhibitor Heavy chain 3 (Itih3) play an important role in cancer and oxidative stress. Smoking of tobacco reduces Itih3 in the plasma and activates hyaluronan signaling through Cd44, but the impact of Cd44 on Itih3 expression is unknown. Here, we studied changes induced by the tobacco component nicotine on the glutathione (GSH) antioxidant system and Itih3 gene expression in Cd44 knockout mice. Cd44 deficiency decreased baseline total GSH and oxidized glutathione (GSSG) levels in the liver compared to wildtype mice. However, contrary to wildtype mice, chronic oral nicotine administration (200 μg/ml) failed to further reduce total GSH and GSSG in Cd44 mice. Sex differences with lowered glutathione levels in females was also detectable only in wildtype but not Cd44 knockout mice. Itih3 mRNA levels in the liver and hypothalamus were not affected by nicotine, Cd44 genotype or sex. Nonetheless, the correlation between Itih3 and total GSH levels in the liver ($r = 0.42$, $p < 0.05$) suggested a role of Itih3 in glutathione metabolism in WT mice. Again this effect was diminished in Cd44 knockout mice. The disappearance of nicotine effects, sex differences and correlations between Itih3 and total GSH in Cd44 knockout mice compared to wildtype animals suggests an interaction between nicotine, Cd44 and/or sex-dependent signaling in the regulation of glutathione metabolism.

1. Introduction

Worldwide cigarette smoking remains one of the leading but preventable causes of death and vascular disease. Combustible tobacco contains free radical species that are damaging to organs [1] and at least 72 known carcinogens [2]. To reduce the harmful effects of tobacco, cigarette smoking is increasingly being replaced by nicotine consumption, which is the major addictive component of tobacco. Direct nicotine delivery methods such as nicotine patches, inhalers, snus and nicotine gums were originally developed as an alternative to tobacco smoking and as aids for quitting smoking. However, the widespread use of new generation nicotine delivery methods such as e-cigarettes and vape pens causes nicotine addiction in adolescents and young adults and can initiate cigarette smoking in the long term [3,4]. Nicotine itself is not completely harmless, because it is metabolized by hepatic enzymes to cotinine and pro-carcinogenic nitrosamino ketones, such as nitrosonornicotine [5]. Nicotine further induces oxidative stress altering the cell's glutathione system, thus potentially making organs more vulnerable to inflammatory diseases such as steatohepatitis and respiratory disease, injury, and cancer [6–8].

Oxidative stress provoked by tobacco smoke induces inflammation and leads to depolymerization of hyaluronan, which can activate the receptor Cluster of Differentiation 44 (Cd44). In turn, the activation of Cd44 signaling can scavenge reactive oxygen species (ROSs), which can promote growth of tumor cells by protecting them from oxidative stress-induced damage [7,9,10]. Inter-alpha-trypsin inhibitor heavy chain 3 (Itih3) is a plasma protease inhibitor produced by the liver, which is also a hyaluronan-binding protein involved in inflammation and cancer. Itih3 stabilizes the extracellular matrix, potentially by preventing depolymerization of hyaluronan [11]. In the plasma of cigarette smokers, Itih3 protein levels were reduced [12]. A role of Itih3 in oxidative stress is further supported by regulation of Itih3 gene

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expression by oxidative stress in organotypic hippocampal cultures, although changes in Itih3 transcript levels were dependent on the subregion investigated [13]. At present it is not known, whether oxidative stress induced by nicotine is sufficient to downregulate Itih3 expression in the liver, which may explain reduced Itih3 levels in the plasma of tobacco smokers, or whether such mechanisms are mediated by activation of Cd44 signaling.

Therefore, a chronic oral nicotine delivery method shown to induce oxidative stress was used to examine whether nicotine downregulates Itih3 gene expression in the liver, which metabolizes about 70% of the orally consumed nicotine during first pass [5,14]. As a measure of oxidative stress, glutathione levels were studied, because glutathione is a key component of the endogenous antioxidant defense system [15]. Furthermore, a potentially protective effect of Cd44 signaling on the glutathione system following nicotine treatment and its role of the regulation of Itih3 gene expression were studied using Cd44 deficient mice.

2. Material and methods

2.1. Mice

B6.129(Cg)-Cd44tm1Hbg/J male and C57BL/6 J female mice were purchased from The Jackson Laboratory (Sacramento, CA, USA). Mice were maintained under a 12-hour light/dark cycle at 22 °C. Standard rodent chow (Harland Teklad, Madison, WI, USA) and water were provided ad libitum. Pups were weaned at 3–4 weeks of age. All procedures were carried out in accordance with the Creighton University IACUC Policies and Procedures (0929.1 and 0934.1). Pups were tail clipped and ear marked for identification at 10–15 days of age using sterile techniques. Genomic DNA was isolated from tail biopsies using the Gentra Puregene Tissue Kit (Qiagen, Germantown, MD, USA). Genotyping was performed using standard PCR with primer sets for wildtype (WT) and Cd44 knockout (KO) alleles (WT_forward GGCGA CTAGATCCCTCCGTT, WT_reverse ACCCAGAGGCATACCAGCTG, KO_forward CTTGGGTGGAGAGGCTATTC, KO_reverse AGGTGAGATG ACAGGAGATC) and agarose gel electrophoresis (WT 175 bp, KO 280 bp).

2.2. Chronic oral nicotine delivery

At the age of six weeks, mice received p.o. either 200 µg/ml (−)-nicotine free base (N3876, Sigma-Aldrich, St. Louis, MO, USA) dissolved in autoclaved tap water (replaced weekly) or autoclaved water only (controls) for the duration of three weeks [14]. The estimated amount of daily nicotine intake was calculated as 29.10 ± 1.54 mg/kg body weight (BW) per mouse, based on 2.83 ± 0.11 ml/day water consume and the BWs recorded at the start and end of the experiment. After three weeks, mice were euthanized using isoflurane and decapitated during the light phase (8:00-12:00). Blood and tissue samples were collected, snap-frozen using liquid nitrogen, and stored at −80 °C until further use.

2.3. Cotinine and glutathione assays

Blood collected in EDTA-coated tubes (Fisher Scientific, Pittsburg, PA) was centrifuged at 6000 rpm (10 min, 4 °C) to obtain plasma supernatant. Cotinine levels were assayed in plasma using the Immunalyis Cotinine Direct ELISA Kit according to the manufacturer's instructions (Immunalysis, Pomona, CA, USA).

Glutathione (GSH) levels were measured in tissue samples of the liver using a kit for colorimetric enzymatic recycling containing glutathione reductase from S. cerevisiae (CS0260, Sigma-Aldrich, St. Louis, MO, USA). The right liver lobe (superior part) was dissected on an ice-cold platform. Samples were deproteinized with 5% 5-sulfosalicylic acid (centrifugation at 10,000 rpm, 4 °C, 10 min). Total (reduced and oxidized) GSH was measured with a kinetic assay based on the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) to 2-nitro-5-thiobenzoic acid and recycling of the oxidized glutathione (GSSG) generated in the reaction by glutathione reductase and NADPH. For measuring GSSG, lysates were incubated with 4-vinylpyridine to block free thiols of reduced GSH (1 h, room temperature). Absorbance was measured at 405 nm (1 min intervals, 10 min) with an EnSpire® Multimode Plate Reader 2300 (Perkin Elmer, Waltham, MA, USA), and net slopes of experimental samples were compared to net slopes of standards using GraphPad Prism 7 software.

2.4. Itih3 mRNA expression

Itih3 gene expression was studied in tissue samples dissected out on an ice-cold platform. The lobe of the right liver (inferior part) as well as the hypothalamus, which is among the brain regions with the highest Itih3 gene expression [16], were used for analyses. Tissue samples were homogenized in lysis buffer and total RNA was isolated (mirVana RNA Isolation Kit with TURBO DNAse; Ambion, Austin, TX, USA). The quality/concentration of RNA was checked using NanoDrop (Wilmington, DE, USA). Total RNA (0.4–1 µg) was reverse-transcribed into cDNA with random hexamer primers using the Superscript III First Strand Synthesis Kit (Life Technologies, Grand Island, NY, USA) in a thermal cycler. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with primers from Integrated DNA Technologies (Coralville, IA, USA) for Itih3 (Forward 5′−CCCGGCGCATTTATGAAGAT-3′; Reverse 5′-ATGGCGTTCTCGGGGTA TTC-3′) and the house keeping gene succinate dehydrogenase complex, subunit A, flavoprotein (SDHA; Forward 5′−CCTGTCCTATGTGGACG TTG-3′, Reverse 5′-GTTTTGTCGATCACGGGTCT-3′). Relative Itih3 gene expression was calculated using crossing point (Cp) values and experimentally determined PCR efficiency for Itih3 and SDHA.

2.5. Statistical analyses

Statistical analyses were performed using IBM-SPSS software 24.0. Multiple groups were compared using a two- or three-way analysis of variance (ANOVA) for the main factors nicotine-treatment, genotype and sex as appropriate. In addition, a four-way ANOVA (treatment x genotype x sex x brain region) with repeated measurements on the last factor (within subject factor) was performed to compare Itih3 gene expression in different tissues (liver and hypothalamus). Correlation analyses were performed using the Spearman Rho test. Data of all WT and Cd44 KO animals, in which glutathione and Itih3 mRNA levels were measured, were included into the study (see Table 1 and Fig. 1 legends for exact numbers). Two-tailed p-values < 0.05 were deemed significant. Data were expressed as mean +/- SEM.

3. Results

3.1. General effects of nicotine treatment

The role of chronic oral nicotine delivery was studied on body weight and plasma cotinine levels (Table 1). Three-way ANOVA (treatment x genotype x sex) revealed a significant increase in plasma cotinine levels in animals that received nicotine, whereas cotinine levels remained below the detection limit of the assay in control animals. Moreover, nicotine-treated animals showed a significant reduction in the gain of their BW during the three week treatment period. The reduction in BW gain was sex-dependent resulting in lower weight gain in males than in females.

3.2. Glutathione levels

As seen in Table 1, a three-way ANOVA (treatment x genotype x sex)

Table 1
Effect of chronic oral nicotine administration on the glutathione system in wildtype (WT) and *Cd44* knockout (KO) mice.

		Plasma cotinine ng/ml	% BW gain	Total GSH liver nmol/ml	GSSG liver nmol/ml	GSSG/Total GSH liver
Wildtype	Control M	< 1.0	16.1 ± 1.5	890.0 ± 44.5	17.9 ± 1.9	0.020 ± 0.002
	Nicotine M	541.5 ± 52.1	12.6 ± 1.8	759.3 ± 39.1	15.0 ± 1.4	0.020 ± 0.002
	Control F	< 1.0	11.5 ± 1.5	741.0 ± 37.0	12.7 ± 1.0	0.017 ± 0.001
	Nicotine F	548.3 ± 51.6	8.0 ± 1.7	580.1 ± 31.7	7.0 ± 1.2	0.012 ± 0.002
CD44	Control M	< 1.0	16.8 ± 2.0	716.2 ± 38.2	10.5 ± 1.1	0.015 ± 0.001
KO	Nicotine M	563.8 ± 56.2	10.3 ± 2.2	809.9 ± 78.8	14.3 ± 2.0	0.018 ± 0.002
	Control F	< 1.0	12.6 ± 2.2	682.8 ± 35.0	11.4 ± 1.8	0.016 ± 0.002
	Nicotine F	498.6 ± 47.3	11.0 ± 2.0	689.9 ± 43.9	9.3 ± 2.0	0.014 ± 0.003
Nicotine Effect		**$F_{57,1}$ = 467.54*****	**$F_{58,1}$ = 8.011****	$F_{44,1}$ = 2.289	$F_{44,1}$ = 2.401	$F_{44,1}$ = 0.602
Genotype Effect		$F_{57,1}$ = 0.076	$F_{58,1}$ = 0.224	$F_{44,1}$ = 0.322	$F_{44,1}$ = 2.556	$F_{44,1}$ = 1.286
Nicotine x Genotype		$F_{57,1}$ = 0.076	$F_{58,1}$ = 0.037	**$F_{44,1}$ = 9.667** **	**$F_{44,1}$ = 5.380** *	$F_{44,1}$ = 1.055
Sex Effect		$F_{57,1}$ = 0.344	**$F_{58,1}$ = 5.652***	**$F_{44,1}$ = 14.559*****	**$F_{44,1}$ = 15.162*****	**$F_{44,1}$ = 4.773***
Nicotine x Sex		$F_{57,1}$ = 0.344	$F_{58,1}$ = 0.825	$F_{44,1}$ = 0.856	$F_{44,1}$ = 3.849	$F_{44,1}$ = 3.234
Genotype x Sex		$F_{57,1}$ = 0.524	$F_{58,1}$ = 1.153	$F_{44,1}$ = 1.914	**$F_{44,1}$ = 4.247***	$F_{44,1}$ = 1.868

Plasma cotinine (nicotine metabolite), body weight (BW), components of the glutathione system presented as mean ± S.E.M. (except plasma cotinine in controls below detection limit of 1 ng/ml), and the outcome of three-way ANOVA (nicotine treatment x genotype x sex). Number of cases included into the study for cotinine assay and % BW gain: WT-Control M = 7, WT-Nicotine M = 10, WT-Control F = 10, WT-Nicotine F = 9, KO-Control M = 10 (cotinine assay n = 9), KO-Nicotine M = 8, KO-Control F = 7, KO Nicotine F = 8. Number of cases used for total (reduced and oxidized) glutathione (GSH) and oxidized glutathione (GSSG) assays and the GSSG/total GSH ratio in the liver: WT-Control M = 6, WT-Nicotine M = 8, WT-Control F = 6, WT-Nicotine F = 7, KO-Control-M = 6, KO-Nicotine M = 7, KO-Control F = 6, KO-Nicotine F = 6. WT wildtype, KO knockout, two-tailed significance levels $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

performed to compare all experimental groups revealed a significant sex effect on total GSH and GSSG levels and the GSSG/total GSH ratio in the liver, the females showing lower levels for all three variables than males. In addition, there was a significant interaction between nicotine-treatment and the Cd44 genotype for total GSH and GSSG levels in the liver. A significant interaction was also found between the factors Cd44 genotype and sex for liver GSSG levels (Table 1).

For further analyses of total GSH and GSSG levels, therefore independent two-way ANOVA (treatment x sex) tests were performed in WT and Cd44 KO mice. In WT mice, chronic oral nicotine administration significantly decreased total GSH levels for about 15–20% ($F_{23,1}$ = 14.521, p = 0.001) and GSSG levels for about 15–45% ($F_{23,1}$ = 10.054, p = 0.004). In contrast, no such effect was evident for total GSH ($F_{21,1}$ = 0.977, n.s.) or GSSH levels ($F_{21,1}$ = 0.229, n.s.) in Cd44 KO mice. The two-way ANOVA (treatment x sex) further indicated that only WT females showed lower levels in total GSH ($F_{23,1}$ = 18.372, $p < 0.001$) and GSSG levels ($F_{23,1}$ = 23.814, $p < 0.001$) compared to WT males. However, in Cd44 KO mice no significant sex differences were evident for total GSH ($F_{21,1}$ = 2.268, n.s.) or GSSG levels ($F_{21,1}$ = 1.299, n.s.). Neither WT nor Cd44 KO mice showed significant interactions between nicotine treatment and sex for total GSH (WT: $F_{23,1}$ = 0.155, n.s.; Cd44 KO: $F_{21,1}$ = 0.722, n.s.) or GSSG (WT: $F_{23,1}$ = 1.131, n.s.; Cd44 KO: $F_{21,1}$ = 2.665, n.s.).

3.3. *Itih3 gene expression*

A highly significant difference between Itih3 mRNA levels in the liver and hypothalamus was confirmed by a four-way ANOVA (treatment x genotype x sex x tissue) by analyzing the within-subject-effect of tissue type ($F_{58,1}$ = 19.534, $p < 0.001$). However, nicotine treatment ($F_{58,1}$ = 0.027, n.s.), genotype ($F_{58,1}$ = 0.027, n.s.) or sex ($F_{58,1}$ = 0.002, n.s.) did not have any significant effect on Itih3 mRNA

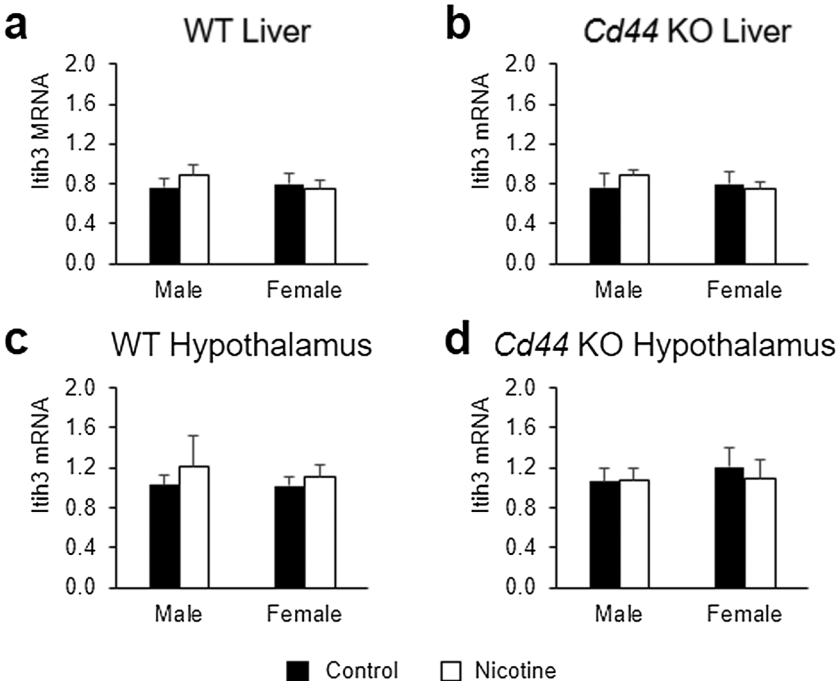

Fig. 1. Itih3 mRNA expression levels presented as mean ± S.E.M. in the liver and hypothalamus. No significant differences were detected between WT and Cd44 KO mice, males and females or following chronic oral nicotine treatment. Numbers of cases included into the study are: WT-Control M = 7, WT-Nicotine M = 10, WT-Control F = 10, WT-Nicotine F = 9, KO-Control M = 10 (cotinine assay n = 9), KO-Nicotine M = 8, KO-Control F = 7, KO Nicotine F = 8.

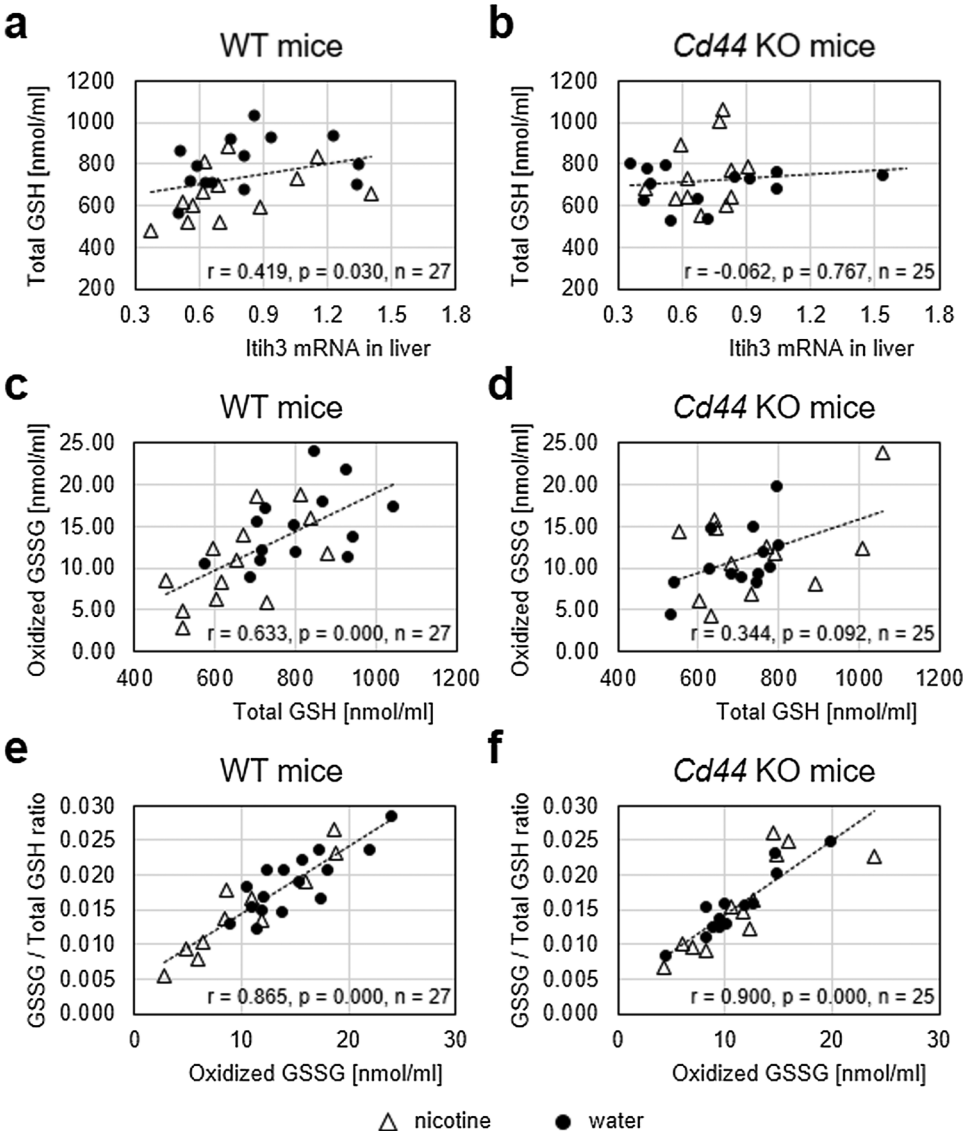

Fig. 2. Significant correlations found in WT and Cd44 KO mice using the Spearman Rho test. GSH glutathione, GSSG oxidized glutathione, r = correlation coefficient, p = significance level, n = number of cases.

levels. Likewise, no significant interactions were observed between the factors treatment, genotype and sex (between-subject-factors) ($F_{58,1}$ = 0.043–1.313). Moreover, the factor tissue (within-subject-factor) did not interact with any of the between-subject-factors ($F_{58,1}$ = 0.004 to 0.161) indicating that the lack of differences seen for the between-subject-factors was applicable to Itih3 mRNA levels in both the liver and hypothalamus (Fig. 1), which was also confirmed in three-way ANOVA analyses (treatment x genotype x sex) separately performed for both tissues (not shown).

3.4. Correlation analyses

The goal of correlation analyses was to detect intraindividual correlations between the glutathione system and Itih3 expression in the liver, which may be independent from nicotine treatment. However, these correlation analyses were performed separately for WT and Cd44 KO mice (Fig. 2) due to the prominent interactions of the genotype with treatment- and/or sex-induced alterations in the glutathione system (see 3.2 above). The Spearman Rho test showed a weak but significant correlation between liver Itih3 mRNA and total GSH levels only in WT ($r = 0.42$, $p < 0.05$) but not in Cd44 KO mice. Similarly, GSSG and total GSH levels only correlated in WT mice ($r = 0.633$, $p < 0.001$), whereas no significant correlations were detected in Cd44 KO mice. Only correlations between GSSG and the GSSG/total GSH ratio were highly significant in both WT and Cd44 KO mice (Fig. 2). Significant correlations were also detected between plasma cotinine and liver GSSG levels in WT mice ($r = -0.383$, $p < 0.05$).

4. Discussion and conclusions

Chronic oral nicotine administration induced a reduction in GSSG and total GSH levels in the liver of WT mice but not Cd44 KO mice, although GSSG and total GSH levels were reduced in Cd44 KO mice. Likewise sex differences seen in the glutathione system of WT mice were not detectable in Cd44 KO mice. Although the Itih3 gene expression itself was not influenced by nicotine-treatment, Cd44 genotype or sex in the liver and hypothalamus, there was a significant correlation between total GSH and Itih3 mRNA levels in the liver of WT but not Cd44 KO mice. The data indicate that the regulation of the glutathione system of the liver by nicotine and sexually dimorphic mechanisms is diminished in Cd44 KO mice. Thus, our data are in favor of an interaction of Cd44 signaling with the nicotine-induced depletion and sex-

dependent regulation of the liver glutathione system.

Using chronic oral nicotine delivery, plasma cotinine concentrations were achieved that were comparable to levels between 250–900 ng/ml found in smokers. The main elimination route for nicotine is through the liver, where the hepatic enzyme CYP2A6 metabolizes nicotine into cotinine and other pro-carcinogenic compounds contributing to the production of free radicals [5]. GSH possesses a cysteine residue, which is readily oxidized to GSSG by electrophilic substances such as free radicals, nitrogen species and ROSs [15]. A GSSG/GSH ratio of < 0.1 is considered a normal redox state for a cell, but oxidative stress can overthrow the cells ability to reduce GSSG to GSH resulting in the accumulation of GSSG, which can be exported out of the cell to be eliminated by other mechanisms, contributing to a net loss of intracellular GSH [17]. Liver levels of oxidized GSSG remained in the range of 1%–5% of total GSH in our study, but the depletion of individual components of the glutathione system by chronic nicotine-treatment is consistent with previous studies [18]. This supports the induction of oxidative stress by nicotine as was previously shown in the kidney following chronic oral nicotine delivery [14].

The rodent glutathione antioxidant system shows marked sex differences, with females showing higher glutathione peroxidase activity, cysteine content and GSH synthesis rate, while males show higher GSH levels, and glutathione transferase and glutathione reductase activity [19]. Therefore, sex differences in hepatic enzyme activity may have contributed to the lower total GSH and GSSG levels observed in females compared to males in WT mice. In addition, secondary metabolic effects caused by chronic nicotine treatment may have contributed to the depletion of GSH and GSSG, e.g., by inducing relative BW loss (reduced weight gain), induction of hypothermia known to drive thyroxin release, and/or induction of liver enzymes metabolizing nicotine [5,20]. However, GSH depletion itself can also influence the rate of metabolism on its own by driving a thermogenic program in adipose cells [21], which may be an additional mechanism that explains some metabolic effects of nicotine.

Baseline total GSH and GSSG levels were lower in Cd44 KO than in WT mice, and the nicotine-induced depletion of total GSH and GSSG was missing, suggesting that Cd44 signaling contributes to the maintenance of glutathione levels and interacts with nicotine-signaling. Consistent with the former observation, a Cd44 splice variant found in cancer was able to stabilize the cystine-glutamate transporter xc(-) subunit (xCT), promoting the uptake of cystine needed to synthesize GSH, thereby contributing to defense against ROS. Likewise, RNAi-mediated ablation of Cd44 reduced the amount of xCT at the plasma membrane, thus depleting intracellular GSH in cancer cells [9,22].

Apart from its roles as an acute phase protein upregulated under inflammatory conditions, a potential tumor suppressor, and a stabilizer of the extracellular matrix due to its capability to bind HA, little is known about Itih3′s function (e.g. in the brain) and the pathways, by which it is regulated [11,23,24]. Itih3 protein levels were reduced in the plasma of cigarette smokers [12] and duroquinone-induced oxidative stress downregulated Itih3 gene expression in the CA3 sector of the hippocampus [13]. However, nicotine treatment did not reduce Itih3 mRNA expression in our liver samples. Should Itih3 serum levels also be reduced in individuals, who use nicotine delivery devices such as e-cigarettes or vape pens, the reduction in serum protein levels of Itih3 observed in tobacco smokers [12] may be the result of wasting or degradation of Itih3, e.g., due to inflammation [23], rather than a reduced Itih3 mRNA synthesis. Itih3 was also reported to be responsive to glucocorticoid treatment [25]. Moreover, nicotine can activate the hypothalamic-pituitary-adrenal axis (HPA) resulting in an increase of glucocorticoid levels, but chronic nicotine delivery leads to development of tolerance dampening the heightened corticosterone release seen after acute treatment [26]. Although Itih3 mRNA levels were not directly affected by nicotine administration, Cd44 genotype or sex, Itih3 transcript levels correlated with total liver GSH in WT mice, suggesting an involvement of Itih3 in glutathione metabolism and/or transport. However, this effect diminished in Cd44 KO mice, adding to the reduced responsivity of the liver's glutathione system to regulation by sex-dependent mechanisms and nicotine in Cd44 deficiency.

Thus, the current results indicate that nicotine exposure for a three weeks period already causes adverse effects on the glutathione antioxidant defense system of the liver. Nicotine interacts with Cd44 signaling and sex-dependent mechanisms in regulating the glutathione system of the liver. However, neither nicotine treatment nor Cd44 signaling seem to be directly involved in the regulation of Itih3 gene expression. Considering the importance of glutathione depletion in development of steatohepatitis and liver cancer [8], long term nicotine intake due to nicotine addiction may be sufficient to make consumers prone to liver disease and to cause detrimental effects on the individual's health.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Transparency document

The Transparency document associated with this article can be found in the online version.

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