Lung function in relation to 2-thiothiazolidine-4-carboxylic acid (TTCA) and Genetic Effect Modification Among Rubber Workers in Sweden

Short title: Lung Function in Rubber Workers

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Abstract

Objective: What is the risk of impaired lung function in contemporary Swedish rubber workers and are there modifying effects of genetic variants? **Methods:** Included in the study were 159 rubber exposed and 118 not-rubber exposed workers. Lung function was analyzed as forced vital capacity percent of predicted and forced expiratory volume in 1 second percent of predicted. Levels of 2-thiothiazolidine-4-carboxylic acid (a marker of carbon disulfide and vulcanization fumes) was assessed with liquid chromatography tandem mass spectrometry. Polymorphisms in glutathione-related genes were analyzed by Taqman-based allelic discrimination and ordinary polymerase chain reaction. **Results:** There was an association between increasing levels of 2-thiothiazolidine-4-carboxylic acid and impaired lung function among exposed workers. The association was modified by glutathione S-transferase alpha 1 (*GSTA1*)-52 and *GSTP1*-114. *GSTM1* had an influence on lung function among unexposed workers. **Conclusions:** There may be a risk of impaired lung function in contemporary rubber workers. Gene-modifying effects may be considered in risk assessments.

Introduction

Workers in the rubber industry are exposed to a complex mixture of toxic compounds, and have an increased risk for different diseases and symptoms (1). Elevated risks of symptoms of the eyes and airways, headache and nausea were recently observed in contemporary Swedish rubber workers by our group (2). Impaired lung function has also been associated with exposure in the rubber industry (3-5). Nevertheless, little is known about lung function among Swedish rubber workers today.

One of the toxic compounds in the rubber industry is carbon disulfide (CS_2), which has been reported to affect many different organs among workers in the rayon industry, although major attention has been paid to the cardiovascular and nervous systems (6). In the rubber industry, CS_2 is formed during the vulcanization process due to decomposition of dithiocarbamates and thiurams (7), which are used as accelerators. In the human body, CS_2 is metabolized to 2-thiothiazolidine-4-carboxylic acid (TTCA), which is excreted in the urine and traditionally has been used as a marker of CS_2 (8, 9). Our group (10) has previously observed elevated levels of urinary TTCA among rubber workers compared to unexposed workers. Since CS_2 are formed during vulcanization, urinary TTCA may serve as an index substance for vulcanization fumes, which is a mixture of more than hundred toxic compounds.

In an earlier study (2), symptoms were found in only a part of the exposed workers; this could partly reflect exposure to different compounds and partly be due to interindividual differences in disease susceptibility. CS_2 may react with glutathione (GSH) through catalysis by the glutathione S-transferase (GST) system (11, 12). Moreover, GSTs are involved in the protection against reactive oxygen, which is a key component in causing damage to the airways by exposure to various toxic substances that are found in the rubber industry (13, 14). GSH-synthesizing genes, as well as GSTs, display genetic polymorphisms, which affect the enzyme function or expression levels (15). Thus, part of the interindividual variation in

disease susceptibility among workers in the rubber industry could be due to genetic variation in GSH-related genes.

The aim of this study was to characterize the risk of impaired lung function among workers in the contemporary Swedish rubber industry, by the use of urinary TTCA as an index substance of CS_2 and vulcanization fumes. Furthermore, the study aimed to elucidate the role of genetic variants in genes, which are involved in the metabolism of and defense against toxic substances present in the vulcanization fumes. For this purpose seven polymorphisms in GSH-related genes (glutamate cysteine ligase catalytic subunit (*GCLC)*-129, glutamate cysteine ligase modifier subunit (*GCLM)*-588, *GSTA1*-52, *GSTM1**O, *GSTP1*-105, *GSTP1*-114, and *GSTT1**O) were selected and analyzed for possible effect modification on lung function. In the present study, interaction is called effect modification.

Materials and Methods

Study Subjects

Included in the study were 159 exposed workers occupied in the vulcanization departments by eight different rubber companies, which are described in detail elsewhere (10). The companies were selected on the grounds of geographical accessibility and diversity of production methods. In addition, 118 unexposed workers were included. They were not occupationally exposed to rubber chemicals but had similar socioeconomic status as the exposed workers. The unexposed workers were assembling armatures at an electric light fittings company (n = 21), meat cutters at a butchery (n = 20), operators at a dairy plant (n = 11), postmen (n = 27) or department store workers (n = 39) and they were working in the same region of Sweden, and in towns of the same size, as the exposed workers. Subjects selected to participate were those who were present at work on the day of the lung function measurements and who had been working for at least half their shifts (4 hours) when the spirometries were performed. Study subjects with Eastern Asian descent (n=21), but no others, were excluded from the study due to expected differences in genotype frequency. Some individual characteristics of the exposed and unexposed workers are shown in Table 1. The study subjects gave their informed written consent to take part in the study. The study was approved by the Regional Ethical Committee of Lund University.

Lung Function

Baseline spirometries were performed on Tuesday through Thursday from June 2001 to June 2003 at the workplaces of the exposed and unexposed workers according to the guidelines of the European Respiratory Society (16). Lung function was measured as forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) on a Vitalograph (Vitalograph, Buckingham, UK). These parameters were compared to reference values and expressed as %

of predicted (FVC%, FEV_1%). For most subjects, the data provided by Berglund et al. (17) for a Swedish population was used for reference, but for a few subjects below the age of 21 years, for whom Berglund et al had no reference, the data provided by Quanjer et al (16), was applied. The Vitalograph was calibrated by the use of a 1-L syringe, according to the guidelines of the European Respiratory Society at least daily and after every transfer of the Vitalograph. Valid FVC values were obtained from 129 exposed and 118 unexposed workers and valid FEV_1 values from 155 exposed and 118 unexposed workers. Some lung function values were missing because those spirometries did not meet the requirements stated for acceptable performances according to Berglund et al (17) and Quanjer et al (16).

Atopy

Atopy was defined as positive when response to at least one allergen in the Phadiatop test [a test measuring the concentration of specific IgE in serum against dog, cat, horse, timothy, birch, mugwort, mite (D.pteronyssinus, D.farinae), mold (Cladosporium), olive, and Parietaria] was observed.

Analysis of TTCA in Urine

Urine was collected both from exposed and unexposed workers during the last 4 hours of an 8-hour work shift on the same day as the spirometries were performed. The main sources of TTCA between exposed and unexposed workers are different (work-related exposure of CS_2 vs endogenous TTCA in cruciferous vegetables) (8, 18). The level of TTCA was analyzed by liquid chromatography tandem mass spectrometry as previously described (19). The limit of detection (LOD) was determined to be 1 ng/mL urine. Samples with a concentration below the LOD were assigned a value of half the LOD. The precision was 11% at 10 ng/mL and 7% at 70 ng/mL. The results were within the tolerance limits in the Round Robin intercomparison

program (Professor Dr Med. Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg). The levels of TTCA were adjusted for creatinine content, which was analyzed enzymatically according to Mazzachi et al (20).

Polymorphisms Analyzed

Seven polymorphisms in GSH-related genes were analyzed. Their functional impacts on enzyme function or expression levels are presented below, and their impact on lung function or TTCA levels, when known.

GCLC contains a polymorphism in the promoter at position –129. The T allele has shown lower promoter activity compared to the C allele in vitro in human endothelial cells (21). The 5'-flanking region of *GCLM* contains a polymorphism (-588C/T) in which the T allele has shown lower promoter activity compared to the C allele when exposed to oxidants (22). Nakamura et al (22) also showed that the plasma GSH levels were significantly lower in CT and TT genotypes than in CC genotype.

Both *GSTM1* and *GSTT1* have nonfunctional null alleles, which are due to deletions of the genes (23). Thus, individuals who have a homozygous deletion have no enzymatic activity. These genotypes are named *GSTM1**O and *GSTT1**O whereas genotypes with at least one functional allele are named *GSTM1**1 and *GSTT1**1. Asthmatic children with *GSTM1**O genotype have been shown to have an ozone-related decrease in lung function, whereas children with *GSTM1**1 had not (24). Furthermore, *GSTT1**O has been associated with accelerated lung function decline in men in the general population (25). We found in a previous study of vulcanization workers that study subjects with *GSTM1**O genotype had lower levels of TTCA compared to subjects with *GSTM1**1, whereas study subjects with *GSTT1**1 genotype had lower levels of TTCA compared to subjects with *GSTT1**O (10)."

GSTA1 contains a polymorphism in the promoter (-52G/A), which may cause differential gene activity as the variant allele misses a binding site for the transcription factor Sp1 (26).

Two functional polymorphisms in the coding region of *GSTP1* were also examined. The variant allele of the polymorphism named *GSTP1*-105 encodes valine (val) instead of isoleucine (ile) at codon 105 due to a base pair exchange where G substitutes A (27). The variant allele of the polymorphism named *GSTP1*-114 encodes valine (val) instead of alanine (ala) at codon 114 due to a base pair exchange where T substitutes C. The variant alleles of *GSTP1*-105 and *GSTP1*-114 may affect the ability and the rate at which different toxic compounds bind to GSTP1-1 (27). The variant allele of *GSTP1*-105 is overrepresented in a variety of cancers (28). It has been, however, found in lower frequency in individuals with atopy or asthma, compared to controls, by some investigators (29, 30), but not by others (31).

The genotype frequencies in the present study are shown in Table 1.

Genotyping

DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) at the DNA/RNA Genotyping Laboratory, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö University Hospital, Malmö, Sweden, or by a modified salting out extraction method (32).

GCLC, GCLM, GSTA1 and *GSTP1* were genotyped with Taqman-based allelic discrimination (ABI 7000; Applied Biosystems, Foster City, CA, USA) as described earlier (10, 33). *GSTM1* and *GSTT1* were genotyped with ordinary polymerase chain reaction and subsequent agarose electrophoresis as described earlier (10, 34, 35).

For all analyses positive controls for the different genotypes and a negative control (water instead of DNA) were included in each run. Moreover, approximately 10% of the samples were reanalyzed as a control of the method.

Statistical Analysis

The exposed workers were compared to the unexposed with respect to FVC% and FEV_1%. Associations between TTCA levels or atopy or smoking and lung function were evaluated separately between the exposed and unexposed workers. Among the exposed workers, possible gene-modifying effects on the association between TTCA and lung function were estimated by analyzing the interaction between the exposure and the independent variable in a separate analysis. Among the unexposed workers, exposure-independent effects of genotype on lung function were investigated.

All analyses were performed using analysis of variance. A probability-probability plot suggested that the levels of TTCA should be transformed (natural logarithm) before use in the models. Thus, the effect estimates obtained by analysis of variance and by using a transformed predictor, were multiplied by ln2 and the resulting product was interpreted as the change in the average value of the outcome for every 100% increase in the predictor (36).

Since the lung function values were adjusted for age and sex by the use of reference values (16, 17), these factors were not considered as potential confounders, but only atopy and smoking were used to adjust the effect estimates. All models were adjusted for these factors unless otherwise stated. Atopy and smoking were considered as both possible effect modifiers and confounders.

Genotypes were dichotomized as follows: *GCLC*-129 and *GCLM*-588 (C/C and C/T + T/T), *GSTA1*-52 (G/G and G/A + A/A), *GSTP1*-105 (ile/ile and ile/val + val/val) and *GSTP1*-

114 (ala/ala and ala/val + val/val). Effects of combined genotypes of *GSTA1*-52 + *GSTP1*-114 and *GSTM1* + *GSTT1* were further analyzed.

For all statistical analyses, SPSS v.13.0 (SPSS Inc, Chicago, IL, USA) was used. For all analyses, two-sided significance was used. Statistical significance refers to $P \leq 0.05$ or, equivalently, to a 95% confidence interval (CI) for a difference that excludes zero.

Results

Exposure and Lung Function

The exposed and the unexposed workers had similar FVC% (95% CI, –4.8, 1.7) and FEV_1% (95% CI, –4.0, 2.3) (Table 2). FVC% and FEV_1%, however, decreased with increasing TTCA levels among the exposed workers; for every 100% increase in TTCA level the FVC% decreased in average 1.2% units (95% CI, –2.3, -0.15) and FEV_1% decreased 0.98% units (95% CI, -1.9, -0.028). The association between TTCA and lung function was neither modified by atopy (95% CI, –2.9, 1.7 regarding FVC%, 95% CI, –2.3, 1.7 (FEV_1%)) nor smoking (95% CI, –1.6, 2.7 (FVC%), 95% CI, –1.2, 2.9 (FEV_1%)). Among unexposed, TTCA had no statistically significant effect on lung function (95% CI, –0.78, 1.7 (FVC%); 95% CI, –0.29, 2.2 (FEV_1%)). The levels of TTCA differed between exposed and unexposed workers. The geometic mean in the exposed cohort was 20 μmol/mol creatinine (range: <LOD, 700; n = 149) and in the unexposed cohort 4.3 μmol/mol creatinine (<LOD, 460; n = 114).

Atopy had a statistically significant effect on FVC%, but not on FEV_1%, in the exposed cohort, but not in the unexposed cohort. Smoking had no effect on lung function among neither exposed nor unexposed workers (data not shown).

Genetic Modifying Effects on Lung Function

In the exposed cohort an gene-modifying effect on the association between TTCA levels and lung function was indicated for *GSTA1*-52 on FVC% and for *GSTP1*-114 on FEV_1%, in that individuals with *GSTA1*-52 (G/G) had decreased risks compared to individuals with *GSTA1*-52 (G/A + A/A) genotypes (P = 0.027) and individuals with *GSTP1*-114 (ala/ala) had decreased risks compared to individuals with *GSTP1*-114 (ala/val + val/val) genotypes (P =

0.034; Table 3). There was no notable difference between adjusted and unadjusted differences (data not shown).

No statistically significant results were found when analyzing combined genotypes (*GSTA1*-52 + *GSTP1*-114 and *GSTM1* + *GSTT1*; data not shown).

Among unexposed workers, individuals with *GSTM1**O genotype had in average 6.5% units lower FVC% and FEV_1% compared to individuals with *GSTM1**1 genotype (95% CI, –11, -1.6 for both lung function parameters). No other statistically significant associations were observed between genotype and lung function (data not shown).

Discussion

In the present study, the results suggested an effect of CS_2, or a covariating exposure, on lung function in rubber workers. In addition, it was indicated that *GSTA1*-52 and *GSTP1*-114 modify the association between TTCA levels and lung function. Among unexposed workers, TTCA had no effect on lung function, whereas individuals with *GSTM1**O demonstrated a risk for decreased FVC% and FEV_1%.

Lung function was studied only as baseline spirometries during workdays in the middle of the week and we found no significant difference in lung function between exposed and unexposed workers. However, since lung function measurements were not performed both before and after exposure (before and after a shift work, a working week, or a vacation) among the participants, and moreover, since no reversibility tests were done, slight obstructive effects from vulcanization fumes may well have been missed. Thus, we may underestimate the risk of the exposure. Furthermore, a selection bias might exist, eg, due to primary or secondary healthy worker selection, or bias in participation. This may have caused an underestimation or overestimation of the risk.

Previous findings indicate an impairment of FEV_1% and FVC% in vulcanization workers (3, 37), and an across-shift decrease in FEV_1% and FVC% at heavy exposure to vulcanization fumes (3). More recently, Zuskin et al (5) found a decline in lung function among smoking rubber workers and a not significant decline among nonsmoking rubber workers, when compared with controls. We found a slight impairment on lung function, but no effect modification by smoking in our study. In the Croatian study (5), the lung function values before shift were lower (FVC% mean 88%, FEV_1% mean 87 to 88%) than those found during work in the present study (FVC% 97%, FEV_1% 98%).

When analyzing the association of TTCA and lung function among exposed workers, increased levels of TTCA were associated with a decrease in FVC% and FEV_1%, although

this was only statistically significant when using TTCA values as a continuous variable. This observation could reflect a direct effect of CS_2 or its metabolites on lung function. Nevertheless, since high TTCA levels may also be an index substance of vulcanization fumes (10), effects of other toxic compounds present in the fumes could play a role. However, the dose-response effect of TTCA on lung function should be cautiously interpreted since the TTCA values were log-transformed in order to fit into a linear model, and moreover, this model may not be true at higher exposure levels.

We found that atopy had an effect on FVC%, but not on FEV_1%, among exposed workers. Nevertheless, we found no modifying effect of atopy on the association between TTCA levels and lung function in this cohort.

GSTs are involved in the protection against reactive oxygen and we found a possible effect modification of *GSTA1*-52 and *GSTP1*-114 on the association between TTCA levels and lung function. GSTA1-1 is present in a wide range of tissues (26). The mean expression of GSTA1-1 in liver samples from *GSTA1* (G/G) donors is approximately 4-fold higher than in samples from *GSTA1* (A/A) donors (38). However, *GSTA1* genotype does not correlate with GSTA1-1 expression in pancreas (39) or GST activity in rectum (40). Since there are no data of effects of *GSTA1*-52 genotype on expression levels or activity in lung tissue, it is difficult to interpret the result from the present study.

In patients with chronic obstructive pulmonary disease, who have an increased oxidative burden, individuals with *GSTP1*-114 (ala/val val/val) genotypes had a significantly decreased FEV_1% as compared to individuals with *GSTP1*-114 (ala/ala) (41). This was not observed among controls or when investigating the *GSTP1*-105 polymorphism in the same study subjects. These data is in agreement with the results from our study.

A possible effect modification of *GSTA1*-52 and *GSTP1*-114 was found in the present study even though the exposure assessment of hazardous chemicals was crude. This indicates

rather large differences between the genotypes. The results, however, need to be confirmed using better exposure assessments.

The gene-modifying effects were further stressed when analyzing the unexposed cohort. We found neither effect of TTCA on lung function, nor any direct effects of *GSTA1*-52 and *GSTP1*-114. However, the *GSTM1**O genotype was associated with impaired FVC% and FEV_1%, which is contrary to what has been observed among adolescents and adults in the general population (25, 42). Nevertheless, in studies on *GSTM1* genotype and exposure, individuals with *GSTM1**O had decreased lung function and increased sensitivity to nasal allergen challenge (24, 43).

In conclusion, the findings demonstrate that rubber workers may be at risk of impaired lung function at contemporary exposure conditions. Moreover, gene-modifying effects ought to be considered when performing risk assessments.

Acknowledgement

The study was supported by AFA [Swedish Labor Market Insurance Company], the Swedish Council for Working Life and Social Research, the Swedish Research Council, Skane county council's research and development foundation, and the Medical Faculty at Lund University, Sweden.

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Table 1 The characteristics and genotype frequencies of the exposed and unexposed workers.

	Exposed (n = 159) *	Unexposed (n = 118)†
Age, median (range) yr	38 (19-65)	41 (19-63)
Sex (male/female)	80 / 79	57 / 61
Smoking; n (%) ‡	53 (33)	44 (37)
Atopy; n (%)	39 (26)	31 (27)
GCLC-129 (C/C); n (%)	122 (82)	97 (85)
(C/T); n (%)	24 (16)	17 (15)
(T/T); n (%)	2 (1.4)	0
GCLM-588 (C/C); n (%)	105 (71)	85 (75)
(C/T); n (%)	37 (25)	23 (20)
(T/T); n (%)	6 (4.1)	6 (5.3)
GSTA1-52 (G/G); n (%)	47 (32)	36 (32)
(G/A); n (%)	78 (53)	56 (49)
(A/A); n (%)	23 (16)	22 (19)
*GSTM1**O; n (%)	77 (53)	49 (43)
*GSTM1**1; n (%)	69 (47)	65 (57)
GSTP1-105 (ile/ile); n (%)	63 (43)	57 (50)
(ile/val); n (%)	75 (51)	46 (40)
(val/val); n (%)	10 (6.8)	11 (9.6)
GSTP1-114 (ala/ala); n (%)	123 (83)	103 (90)
(ala/val); n (%)	24 (16)	11 (10)
(val/val); n (%)	1 (0.7)	0

*GSTT1**O; n (%)	26 (18)	17 (15)
*GSTT1**1; n (%)	119 (82)	97 (85)

* The atopy status was known for 150 exposed workers and the genotype for 145-148 exposed workers depending on the polymorphism.

† The atopy status was known for 113 unexposed workers and the genotype for 114.

‡ Study subjects were denoted smokers if they stopped smoking less than 6 months ago.

Table 2 Mean (range) of FVC% and FEV_1% among unexposed and exposed workers.

Lung function	Exposure	
	Unexposed	Exposed
FVC%; mean (range)	97 (50 – 129)	95 (57 – 129)
FEV_1%; mean (range)	98 (45 – 129)	98 (63 – 135)

FVC% denotes forced vital capacity as percent of predicted, and FEV_1% denotes forced expiratory volume in one second as percent of predicted.

Table 3 The relation between urinary levels of TTCA and the risk of changed FVC% and FEV_1% among exposed workers when stratifying for different genotypes.

		FVC%		FEV_1%	
Gene	Genotype	Diff.*	95% CI	Diff.*	95% CI
GCLC-129	C/C	-1.1	-2.3, 0.017	**-1.1**	-2.1, -0.088
	C/T T/T	-1.0	-4.4, 2.4	-0.29	-3.4, 2.8
GCLM-588	C/C	-0.45	-1.7, 0.83	-0.52	-1.7, 0.6
	C/T T/T	**-3.1**	-5.0, -1.1	**-1.9**	-3.7, -0.14
GSTA1-52	G/G	0.79	-1.4, 3.0	-0.23	-2.0, 1.5
	G/A A/A	**-1.8**	-3.1, -0.53	-1.1	-2.4, 0.10
GSTM1	1	-0.65	-2.3, 0.98	-0.88	-2.4, 0.66
	O	**-1.7**	-3.3, -0.13	-0.86	-2.2, 0.47
GSTP1-105	ile/ile	-1.5	-3.1, 0.19	-0.29	-1.7, 1.2
	ile/val val/val	-0.98	-2.4, 0.46	**-1.4**	-2.7, -0.14
GSTP1-114	ala/ala	-0.82	-2.1, 0.41	-0.38	-1.5, 0.71
	ala/val val/val	-2.2	-4.8, 0.36	**-2.2**	-4.3, -0.15
GSTT1	1	**-1.3**	-2.5, -0.056	**-1.2**	-2.3, -0.15
	O	-0.21	-2.8, 2.4	0.71	-2.2, 3.6

* Change in the average value of the outcome for every 100% increase in TTCA level.

The model was adjusted for atopy and smoking. Statistically significant ($P \leq 0.05$) values are indicated in bold. TTCA denotes 2-thiothiazolidine-4-carboxylic acid, FVC% denotes forced vital capacity as percent of predicted, and FEV_1% denotes forced expiratory volume in one second as percent of predicted.