Stress Repression in Restrained Rats by (*R*)-(−)-Linalool Inhalation and Gene Expression Profiling of Their Whole Blood Cells

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As an attempt to quantitatively analyze the physiopsychological effects elicited by odorants, white blood cells and gene expression were profiled in the whole blood of the rats exposed to (*R*)-(−)-linalool during restraint stress for 2 h. In neutrophils and lymphocytes, significant changes caused by the restraint were repressed by their exposure to the odorant. This indicates that inhalation attenuates stress-induced changes. Significant changes on the stress-induced variations were induced by inhalation in 115 gene expression levels. Of those, 109 genes were down-regulated, whereas the remaining 6 were up-regulated. These findings show that (*R*)-(−)-linalool inhalation represses stress-induced effects on the profiles of both blood cells and gene expression. Furthermore, these results suggest the possibility that the odorant-induced effects can be quantitatively evaluated by analyzing the profiles of blood cells and gene expression.

INTRODUCTION

Odorants are low molecular weight compounds that offer important information about environments and exogenous substances including foods. It has been empirically known from ancient times that some odorants bring about psychophysiological effects, such as sedative, stimulative, antistress, anti-inflammatory, and anticonvulsant effects; for example, an antidepressant effect is provided by lemon odor (*1*) and a sleep-enhancing effect by valerian (*2*).

Linalool (3,7-dimethyl-1,6-octadien-3-ol), a monoterpene compound with a floral scent, has been identified in numerous foods and flowers, including tea (*3*, *4*), orange (*5*, *6*), grape (*7*), mango (*8*), lemon (*9*), tomato (*10*), basil (*11*), and lavender (*12*). Its characteristic odor is important not only in the formulation of a variety of fruit flavors and fragrances but also in eliciting certain kinds of psychophysiological effects to which a relatively large numbers of studies have been directed. It has been suggested that the effects are elicited by its actions on the central nervous systems (*13−15*). Two optical isomers of linalool with (*R*)- and (*S*)-configurations act differently on psychophysiological parameters (*16*). (*R*)-Linalool has been reported to elicit a significant decrease in heart rate under stressed conditions because of having a sedative effect (*17*).

In recent years, there has been more interest in the psychophysiological effects elicited by odorants (described above) because they can be reasonably expected to contribute to health maintenance and promotion. However, research assessing these effects

of inhaled odorants in vivo is still quite limited. Moreover, the effects are considered to be caused by complexes of psychological and physiological aspects (*13−17*). Therefore, we tried to objectively quantify the effects the inhaled odorant has in vivo by multidisciplinary profiling of stress hormones, blood cells, and gene expression. For this purpose, we focused on differences in whole blood obtained from normal Wistar rats exposed to a 2 h restraint, which was defined as a combination of physical and psychological stressors, with or without exposure to the odorant.

MATERIALS AND METHODS

Animals. Male Wistar rats aged 7−8 weeks were housed in a room maintained on a light/dark cycle with lights turned on at 9:00 a.m. and off at 9:00 p.m., food and water available ad libitum. After the acclimatization to the environment and to the investigators for 1 week, 12 rats were divided into three groups ($n = 4$ for each group): group A was exposed to neither stress nor odor as a control; group B was exposed to stress only; group C was exposed to both stress and odor.

Stress and Odor Inhalation. Rats in groups B and C were stressed for 2 h (from 10:00 a.m. to 12:00 p.m.) by being placed in a restraining plastic tube; they were able to breathe freely but unable to turn around head to tail. During the 2 h restraint, the rats in group C were exposed to odor. Twenty microliters of (*R*)-(−)-linalool (92% ee) was evaporated and allowed to spread throughout the 40 L box in which the rats within the tubes were placed. Immediately after the 2 h, all 12 rats were quickly sacrificed by decapitation, and then their blood samples, 10−15 mL, were collected in EDTA-coated tubes. Two milliliters of blood from each rat was used for DNA microarray analysis, another 1 mL was used for blood cell counting, and the remainder was centrifuged to obtain plasma for the following stress hormone assay.

Blood Cell Count and Stress Hormone Assay. The total numbers and differential counts of leukocytes were measured by an automated

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hematology analyzer (Celltac, MEK-6300; Nihon Kouden, Tokyo, Japan) and manual microscopy. Plasma adrenocorticotropic hormone (ACTH) levels were quantified by an immunoradiometric assay using an ACTH IRMA Yuka kit (Mitsubishi Chemical, Tokyo) and corticosterone levels by a solid phase immunoassay using a Coat-A-Count Rat Corticosterone kit (Diagnostic Products, Los Angeles, CA), respectively, performed at the SRL Medisearch, Inc., laboratories (Tokyo, Japan). The protocols were in accordance with the manufacturers' specifications. Between-group differences were examined for statistical significance using Student's *t* test. A value of $p < 0.05$ was considered to be significant.

DNA Microarray Analysis. Total RNA was isolated from the whole blood in each rat using a RiboPure Blood kit (Ambion, Austin, TX), and then globin mRNA was removed with a GLOBINclear-Mouse/Rat kit (Ambion) (*18*). The quality and quantity of total RNA were assessed by agarose gel electrophoresis and spectrophotometry. A MessageAmp II-Biotin Enhanced SingleRound aRNA Amplification Kit (Ambion) was used to obtain biotinylated cRNA. Briefly, 2 μg of purified total RNA was used to synthesize cDNA, and then biotinylated cRNA was transcribed, fragmented, and hybridized to an Affymetrix Rat Genome 230 2.0 GeneChip. Microarray analysis was performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). After hybridization at 45 °C for 16 h, the array was washed and labeled with phycoerythrin, and the fluorescent signals were scanned using the Affymetrix Gene Chip System. The resultant data were analyzed with Affymetrix software (Microarray Suite 5.0). The arrays in this study had an average percent present call of 35.6 ± 2.9. For data analysis, Genespring software (Silicon Genetics, Inc., Redwood City, CA) was used for normalization, clustering, statistical analyses, and visualization. To correct for the minor differences in the amount of each cRNA applied to the microarrays, the obtained raw expression values were normalized to the 50th percentile per chip. In addition, the procedure "Per Gene: Normalize to specific samples" was applied as an additional normalization step, where the signal for each gene was normalized to the median of group A. For further analysis, filtering criteria based on "detection call" (>75% "present" or "marginal" call in at least one group) was applied to remove data that were not reliably detected. Then, 12263 probe sets of a total of 31099 were used. Between-group differences were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's test. A value of $p < 0.05$ was considered to be significant.

RESULTS

Effects of (*R*)-(−)-Linalool Inhalation on Blood Cells and Stress Hormones under Stressed Condition. To verify the effect of the odor inhalation under the stressed condition, we compared the monitored stress hormone levels and the white blood cell counts in the collected blood between the three treatment groups.

First, we compared the data from the restrained rats (group B) against those from the controls (group A) to verify the effect of the restraint on the blood parameters. The concentration of plasma ACTH was confirmed to be increased from 95 ± 77 to 422 ± 255 pg/mL and that of plasma corticosterone from 43 ± 16 to 373 ± 113 ng/mL (**Figure 1a**), and the percentage of neutrophils was increased from 22.8 ± 5.3 to $45.0 \pm 16.5\%$ (**Figure 1b**). Conversely, the total number of leukocytes decreased from 54500 ± 9434 to 42750 ± 10905 counts/μL and the percentage of lymphocytes from 74.0 ± 5.7 to $51.3 \pm 15.7\%$, respectively (**Figure 1b**). These results were consistent with the earlier reports assessing the effects of restraint stress (*19−21*), suggesting that the stressed state was successfully induced in our experiment.
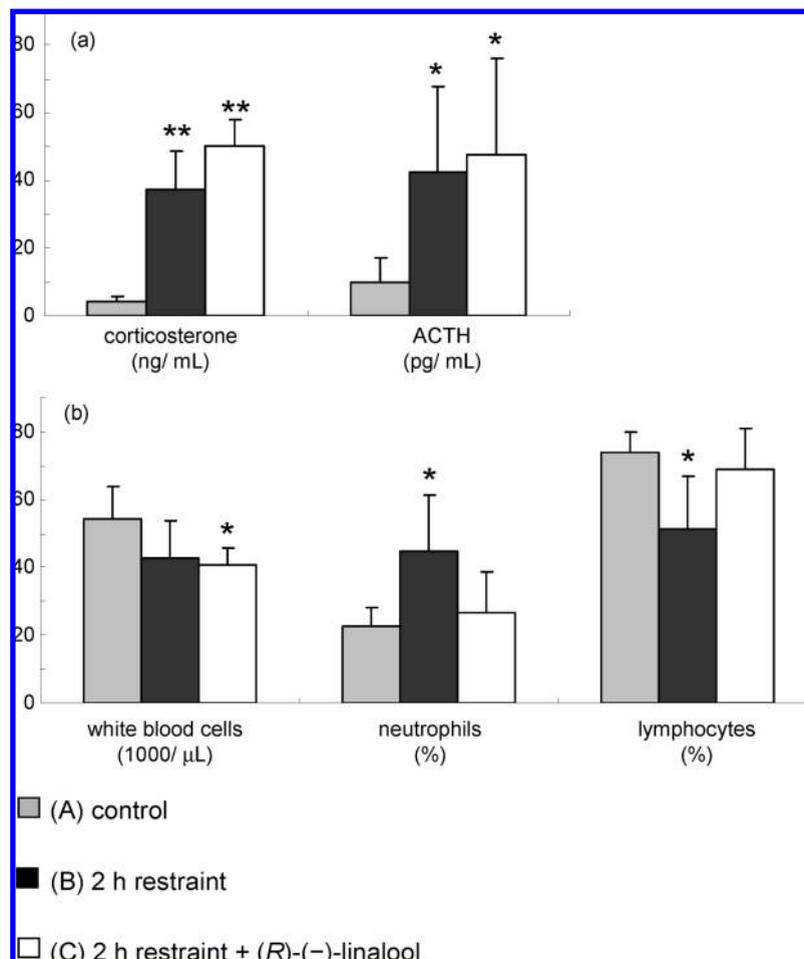

Figure 1. Effects of (*R*)-(−)-linalool on (**a**) plasma ACTH and corticosterone levels and (**b**) leukocyte populations in stress model rats. Data are expressed by means ± S.D. of four rats. * $p < 0.05$, ** $p < 0.01$ compared to the control (Student's *t* test).

Next, we compared the data from the rats that were allowed to inhale (*R*)-(−)-linalool during the restraint (group C) against those from the controls (group A) to investigate the effect of the odor inhalation on the blood parameters. The plasma ACTH and corticosterone levels were found to increase significantly (**Figure 1a**); in contrast, the total number of leukocytes decreased by inhalation during the restraint (**Figure 1b**). No significant change between groups was observed for the percentages of neutrophils and lymphocytes (**Figure 1b**). This result suggests that the changes induced by the restraint stress tend to be suppressed by inhalation of (*R*)-(−)-linalool and also that (*R*)-(−)-linalool recovers the levels of both neutrophils and lymphocytes to their normal levels.

Effects of (*R*)-(−)-Linalool Inhalation on Gene Expression Profile. To address the question of whether it would be possible to detect the effect of (*R*)-(−)-linalool on the differential patterns of mRNA expression in whole blood, we compared holistic changes in the mRNA expression among the three treatment groups. For the analyses, the set of 12263 probes that passed the applicable criteria were used .

First, one-way ANOVA identified 1695 probe sets in which the mRNA levels were found to be statistically different among the three groups. Heat mapping and hierarchical clustering were then performed on the basis of the data of these 1695 probe sets. In the condition tree, the samples of the 12 rats were primarily noted to be clustered into two different subtypes. One included groups B and C (with restraint), whereas the other contained A (without restraint) (**Figure 2**). In addition, the samples of group C formed a different cluster from that of group B (**Figure 2**). These results indicate that the inhaled (*R*)-(−)-linalool has an influence on the gene expression profiles in the restrained rats, although its influence was weaker than that induced by the restraint. In the gene tree, the 1695 genes were broadly classified into six classes according to their between-group differential expression patterns, namely, class 1, where the expression values in group A are lower than in groups B and C; class 2, where values in group C are higher than in groups A and B; class 3, where values in group A are higher than in groups B and C; class 4, where values in group B

are lower than in groups A and C; class 5, where values in group B are higher than in groups A and C; and class 6, where values in group C are lower than in groups A and B. The variation of gene expression in class 4 correlated to the percentage of lymphocytes, and similarly that in class 5 correlated to that of neutrophils. Also, the variation in class 1 correlated to the plasma ACTH and corticosterone levels.

To verify the odorant-induced effects on the stress-altered gene expression profiles, we statistically examined the genes that were differentially expressed between groups A and B, plus B and C, by using Tukey's test, which was applied as a post hoc test after one-way ANOVA. The results showed that the applied restraint significantly altered the expression levels of 696 genes, among which 115 were significantly altered by the inhalation (**Table 1**). These results observed by both clustering and statistical analysis indicate that the inhalation actually altered the gene expression profiles in the whole blood of the restrained rats.

To further assess and clarify the influence of inhaled (*R*)-(−)-linalool on the stress responses induced by the restraint, we examined the expression patterns of the 115 genes that had been significantly altered by the inhalation. As a result, we noted

Table 1. Intergroup Comparisons of Gene Expression Levels between Groups A and B or Groups B and C[a]

	ANOVA	Tukey's post hoc test		
		A vs B	B vs C	A vs B and B vs C
class 1	360	192	21	7
class 2	98	0	55	0
class 3	740	351	50	12
class 4	95	55	62	30
class 5	201	96	154	64
class 6	201	2	149	2
total	1695	696	491	115

[a] The number of probe sets that passed $p < 0.05$ using one-way ANOVA followed by Tukey's post hoc test is shown.
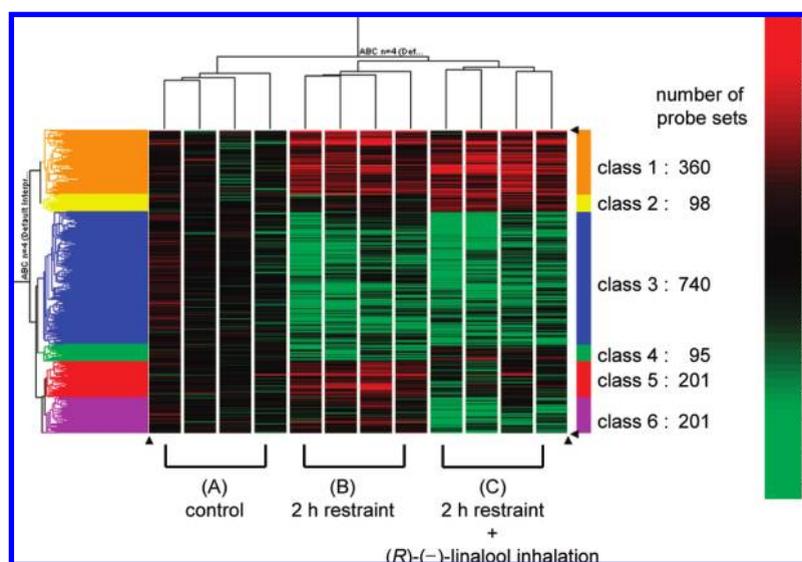

Figure 2. Hierarchical cluster analysis performed on the 1695 stress and/or odorant responsive genes that passed the filtering criteria. The dendrogram was based on whole blood gene expression of the three study groups. The correlation coefficient was used as distance metric with average linkage used as the clustering algorithms. In the heat map, the color indiacts high (red) and low (green) relative levels of gene expression, compared with the median for group A. In the gene tree, the color indicates the cluster name, namely, class 1 where the expression values in group A are lower than group B and C, class 2 where values in group C are higher than group A and B, class 3 where values in group A are higher than group B and C, class 4 where values in group B are lower than group A and C, class 5 where values in group B are higher than group A and C, and class 6 where values in group C are lower than group A and B.
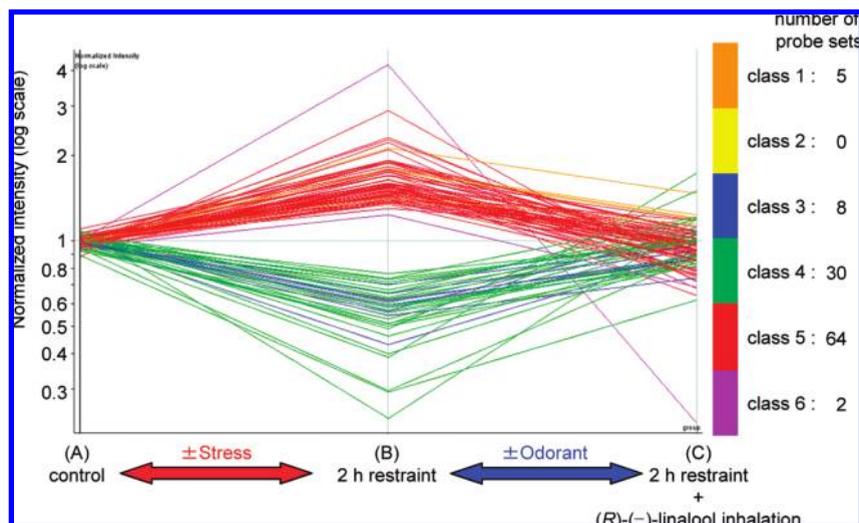
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Figure 3. Variation of the 109 gene expression profiles for which the stress-induced changes were repressed by (*R*)-(−)-linalool. Each line plot shows 2 h restraint and/or (R)-(−)-linalool-induced changes. The 109 gene expression values passed the filtering criteria of both $p < 0.05$ between A vs B and B vs C by Tukey's post hoc test. The color of the lines shows the cluster name, i.e., class 1 to 6, in **Figure 2**.

Figure 4. Variation of the six gene expression profiles for which the stress-induced changes were enhanced by (*R*)-(−)-linalool. Each line plot shows 2 h restraint and/or (R)-(−)-linalool-induced changes. The six gene expression values passed the filtering criteria of both $p < 0.05$ between A vs B and B vs C by Tukey's post hoc test. The color of the lines shows the cluster name, i.e., class 1 to 6, in **Figure 2**.
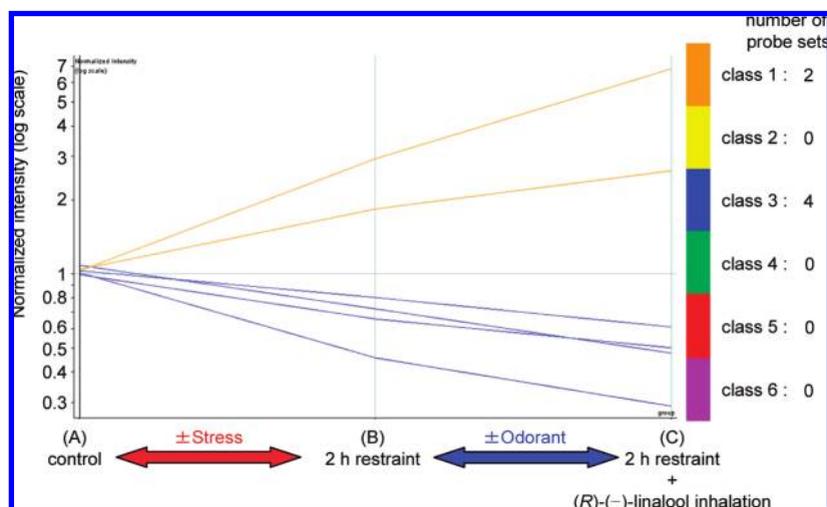
that (*R*)-(−)-linalool inhalation during the restraint significantly repressed the restraint-induced changes in the expression levels of 109 genes (**Figure 3**), whereas it enhanced those of the remaining 6 genes (**Figure 4**). Of the 115 genes, 41 genes having *p* values of <0.01 between groups A and B in the expression levels are shown in **Table 2**. Among the 41 genes, (*R*)-(−)-linalool inhalation repressed the restraint-induced changes in the expression levels of 38 genes, whereas it enhanced those of the remaining 3 genes. These findings suggest that (*R*)-(−)-linalool has a partially repressive effect on changes induced by the stress in the gene expression levels to restore them to their normal levels.

DISCUSSION

The present study is an attempt to quantitatively analyze the psychophysiological effects of odorants. For this purpose, we profiled stress hormones, blood cell counts, and gene expression in the whole blood of restrained rats that inhaled the vapors of (*R*)-(−)-linalool. This profiling revealed a repressive effect by (*R*)-(−)-linalool on the stress-induced changes in blood cells and gene expression levels.

Neutrophils and lymphocytes play important roles in biological defense mechanisms, and their distributions in the blood are controlled by the hypothalamic−pituitary−adrenal (HPA) axis and autonomic nervous system (*22−25*). Aromatherapy massage with linalool-containing essential oil has been documented to bring about a significant increase in peripheral blood lymphocytes (*26*), which were presumed to be under parasympathetic control (*24*). Intriguingly, our results showed that the percentage of lymphocytes was increased by (*R*)-(−)-linalool inhalation in the restrained rats. These observations indicate the possibility that (*R*)-(−)-linalool acts on immune cell distributions via the acceleration of parasympathetic nerve activity. Moreover, the method for profiling the immune cell distributions can be effective in the objective evaluation of the physiopsychological effects induced by odorants. However, the plasma ACTH and the corticosterone levels, which are generally used as biomarkers for assessing the degree of stress state, were not repressed by the inhalation. Because corticosterone mediates the negative feedback that controls excessive responses to stressors (*27*), their secretions play important roles in biological defense.

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Table 2. 41 Genes Showing the Effect of (R)-(−)-Linalool on Stress-Regulated Gene Expression

GenBank Accession no.	symbol	cluster[a]	A vs B fold change[b]		B vs C fold change[b]		p value[c]
			B/A		B/C		
AJ311952	Mrga10	class 1	2.16	↑	1.45	↓	0.022
AF310076	F2rl2	class 1	1.84	↑	1.50	↓	0.029
AI171676	Fhl3	class 1	1.75	↑	1.44	↓	0.003
BF283340		class 1	1.72	↑	1.49	↓	0.037
X02231	Gapdh	class 1	1.43	↑	1.26	↓	0.040
AW141270		class 5	3.27	↑	4.03	↓	0.003
NM_053986	Myo1b	class 5	2.41	↑	3.09	↓	0.002
BI285718		class 5	2.00	↑	1.76	↓	0.018
AW915049		class 5	1.90	↑	2.07	↓	0.004
BG380297	Hfe	class 5	1.88	↑	1.74	↓	0.005
AI111605	Pfkm	class 5	1.72	↑	1.69	↓	0.003
NM_022950	C1galt1	class 5	1.64	↑	1.45	↓	0.017
BF388414	Kcnrg	class 5	1.61	↑	1.72	↓	<0.0005
AI408482	Aph1b	class 5	1.59	↑	1.53	↓	0.003
BE103745	Sort1	class 5	1.57	↑	1.79	↓	0.002
BF416970		class 5	1.55	↑	1.68	↓	0.003
NM_130413	Scap2	class 5	1.54	↑	1.42	↓	0.024
BM392070		class 5	1.49	↑	1.83	↓	<0.0005
BF542593	Tm7sf1	class 5	1.45	↑	1.37	↓	0.016
BF399517		class 5	1.43	↑	1.53	↓	0.002
BI295127	RGD1562938	class 5	1.43	↑	1.23	↓	0.008
BI274399	Loxl3	class 5	1.42	↑	1.47	↓	0.002
AA859922	RGD1308828	class 5	1.34	↑	1.43	↓	0.001
BE104535		class 5	1.30	↑	1.21	↓	0.037
			A/B		C/B		
AA858645	Chd1l	class 3	1.84	↓	1.35	↑	0.028
BI303073		class 3	1.67	↓	1.46	↑	0.006
AI228076		class 3	1.61	↓	1.40	↑	0.012
BF400575	LOC360568	class 3	1.52	↓	1.42	↑	0.018
AI548117	Col10a1	class 4	4.61	↓	5.05	↑	0.003
BF283924	Lamp2	class 4	3.03	↓	2.11	↑	0.020
AA955213	RGD1309362	class 4	2.55	↓	2.20	↑	0.012
BI282363	RGD1311709	class 4	2.18	↓	1.98	↑	0.011
AF223951	Timm22	class 4	1.92	↓	1.63	↑	0.021
BE097233	Polr3h	class 4	1.85	↓	1.62	↑	0.004
BG376705	Ccdc2	class 4	1.83	↓	1.53	↑	0.043
AI012955	Galm	class 4	1.73	↓	1.43	↑	0.015
AI102995	Dhx30	class 4	1.66	↓	1.46	↑	0.030
AI071152		class 4	1.66	↓	1.36	↑	0.015
			B/A		C/B		
U54791	Cxcr4	class 1	1.77	↑	1.42	↑	0.013
			A/B		B/C		
BG153342	Cirbp	class 3	2.22	↓	1.58	↓	0.022
BI278800	Mcrs1	class 3	1.52	↓	1.31	↓	0.013

[a] The cluster name indicates class 1, where the expression values in group A are lower than in groups B and C; class 2, where values in group C are higher than in groups A and B; class 3, where values in group A are higher than in groups B and C; class 4, where values in group B are lower than in groups A and C; class 5, where values in group B are higher than in groups A and C; and class 6, where values in group C are lower than in groups A and B. [b] The fold change was calculated from log ratio obtained by MAS 5.0 software. [c] Statistical analysis was performed between groups A and B or between groups B and C with one-way ANOVA followed by Tukey's post hoc test. The 41 gene expression values passed the criteria of both $p < 0.01$ between groups A and B and $p < 0.05$ between groups B and C including the final 3 genes for which fold changes indicate the enhancement of stress by (R)-(−)-linalool. (A) Control animals that were not exposed to 2 h restraint or (R)-(−)-linalool; (B) animals that were exposed to 2 h restraint; (C) animals that inhaled (R)-(−)-linalool during a 2 h restraint.

Although the hormones remained at stressed levels for 2 h from the start in this study, it is a remarkable feature of the effect by (R)-(−)-linalool that the percentage of both neutrophils and lymphocytes were recovered to their normal levels by the inhalation.

Our microarray studies revealed that stress-induced changes in 109 genes were significantly repressed by (R)-(−)-linalool, although in contrast the changes in 6 genes were enhanced. This is the first study to report the in vivo effect of inhaled odorant by monitoring whole blood gene expression. Moreover, it is of interest that the effects of inhaled odorants can be observed as significant alterations in gene expression profiles. Our findings here, therefore, are noteworthy in demonstrating that the profiling of whole blood gene expression is an effective method for assessing odorant-associated biological processes in vivo.

A strong correlation was observed between distributions of immune cells and expression levels of the 109 genes in which (R)-(−)-linalool repressed the stress-induced changes. Probably, this is because gene expression in whole blood reflected the heterogeneity of the cell populations. It has been suggested that the patterns of gene expression in leukocytes, including neutrophils and lymphocytes, are specific to cell types (28−30). Therefore, the variation in the expression levels of the cell-specific transcripts may have been caused simply by the changes in proportions of blood cell subsets in whole blood. Meanwhile, we found in this study that there also existed various patterns of gene expression and that many of them showed no correlation with the kinetics of neutrophils and lymphocytes. These findings indicate that the gene expression profiles in whole blood might reflect not only the changes of the cell populations but also other influences, such as the regulation of transcription at a cellular level caused by odor inhalation or restraint. Elucidation of the effect of odorants on transcription at a cellular level will be of interest because it will provide deep insights into the mechanisms of physiopsychological effects of odorants.

In conclusion, we found repressive effects of (R)-(−)-linalool on stress-induced changes in both blood cells and gene expression with our method targeted to analyze the components in whole blood. Given that blood samples are the most easily accessible body compartments in humans as well as animals, our method would be easily applied to those tests in humans. Therefore, it is expected that our findings here could largely contribute as a new method to evaluate in vivo effects caused by odorants.

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