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Expression of mRNA in the frontal cortex and hypothalamus in a rat model of acute carbon dioxide poisoning

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ABSTRACT

Acute carbon dioxide (CO_2) poisoning causes no specific features that are revealed upon autopsy, and the pathophysiological mechanism of this syndrome is unclear. To address this issue, in the present study, we exposed rats to CO_2 concentrations ranging from 10% to 60% and determined the effects on mRNA expression. According to the results of Gene Ontology (GO) and cluster analyses of microarrays data, we selected the following genes for further analysis: alkylglycerone phosphate synthase (*Agps*), hypocretin (*Hcrt*), tyrosine hydroxylase (*Th*), heat shock protein beta 2 (*Hspb2*), and opioid receptor delta 1 (*Oprd1*) expressed in the frontal cortex and renin (*Ren*), pancreatic polypeptide (*Ppy*), corticotropin releasing hormone receptor 2 (*Crhr2*), carbonic anhydrase 1 (*Car1*), and hypocretin receptor 1 (*Hcrtr1*) expressed in the hypothalamus. We found significant differences between the expression levels of *Agps* and *Hspb2* mRNAs in the frontal cortex and that of *Ppy*, *Crhr2* mRNAs in the hypothalamus in the presence of high concentrations of CO_2. Further investigation of these genes may clarify the pathophysiology of acute CO_2 poisoning and facilitate the development of novel forensic tests that can diagnose the cause of death.

1. Introduction

Carbon dioxide (CO_2) is a colorless and odorless gas that represents 0.03% of the constituents of air. CO_2 poisoning causes headache, dizziness, nausea, tremor, and fainting [1–3]. In forensic casework, we encounter deaths caused by acute CO_2 poisoning from exposure to gaseous or solid (dry ice) CO_2. However, because acute CO_2 poisoning does not exhibit specific characteristics upon autopsy, except for the congestion of organs [4,5], the cause of death is sometimes mistakenly attributed to hypoxia induced by suffocation.

The details of the mechanism and pathophysiology of CO_2 poisoning are not understood in detail. We do know that exposing rats to CO_2 gas in the absence of oxygen (O_2) rapidly induces acidosis and terminates breathing, suggesting that respiratory acidosis causes the ensuing fatal arrhythmia [6]. In contrast, CO_2 poisoning is induced in dogs exposed to 20% O_2 and 80% CO_2 gas, which suppresses respiration and circulation by inhibiting the activity of the central nervous system and by enhancing the effects of parasympathetic nerve activity [7].

To better understand the pathophysiology of CO_2 poisoning, we developed a rat model of acute CO_2 poisoning by exposing rats to a range of CO_2 gas concentration (10–60%). We then analyzed the levels of gene expression in the frontal cortex and hypothalamus.

2. Materials and methods

2.1. Rat model of acute CO_2 poisoning

The Animal Care and Use Committee of Kyushu University approved the experiments using rats (Nos. A24-225-0, A26-003-0). Wistar rats (eight males, 8-weeks-old, body weight range 301–360 g) (Kyudo Co., Ltd., Saga, Japan) were anesthetized using intraperitoneal injection of medetomidine (0.15 mg/kg) (Domitol, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan), midazolam (2 mg/kg) (Dormicum, Sandoz Co., Ltd., Tokyo, Japan), and butorphanol (2.5 mg/kg) (Vetorphale, Meiji Seika Pharma Co., Ltd.,

Abbreviations: *Agps*, alkylglycerone phosphate synthase; *Car1*, carbonic anhydrase 1; CRH, corticotropin releasing hormone; *Crhr2*, corticotropin releasing hormone receptor 2; FDR, False Discovery Rate; GO, Gene Ontology; *Hspb2*, heat shock protein beta 2; *Hcrt*, hypocretin; *Hcrtr1*, hypocretin receptor 1; NPY, neuropeptide Y; *Oprd1*, opioid receptor delta 1; *Ppy*, pancreatic polypeptide; *Ren*, renin; SAM, significant analysis of microarrays; *Th*, tyrosine hydroxylase.

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Tokyo, Japan). After confirming anesthesia, the rats were placed in a restrainer, and four were exposed to 40% CO_2 (O_2, 20%; N_2, 40%) for 15 min using a nose-only inhalation apparatus (Shin Factory, Fukuoka, Japan) (experimental group). The four rats in the control group were allowed to breathe air normally for 15 min. Cervical dislocation was used to sacrifice all rats. The CO_2 concentration and exposure time were chosen according to published results to allow the expression of detectable levels of mRNAs [1,7].

2.2. Selection of picking region

We selected the frontal cortex because it mediates recognition and judgment, and because the symptoms of mental disorders are exhibited by patients with acute CO_2 poisoning [1,2]. The hypothalamus was selected, because it controls the medulla of vital autonomic nerves that are required for circulation, respiratory movement, and homeostasis.

2.3. RNA isolation and reverse transcription

The frontal cortex and hypothalamus were immediately dissected after cervical dislocation. Tissues were homogenized using a Bio Mashe (Nippi Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions, and total RNA was extracted using a Maxwel 16 System (Promega Corporation, Madison, WI, USA) and a Maxwell 16 LEV simply RNA Tissue Kit (Promega Corporation). Total RNA (1000 ng) was utilized as a template, and complementary DNA (cDNA) was synthesized using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and a High Capacity RNA-to-cDNA kit (Applied Biosystems).

2.4. Microarray and data analysis

The cDNAs transcribed from the frontal cortex and hypothalamus RNAs were hybridized with 44,256 probes included in the oligo DNA tip (Agilent Technologies, Santa Clara, CA, USA). After eliminating control probes, overlapping probes and probes that detect significant decreases in expression levels, 24,305 and 25,089 were extracted from the frontal cortex and hypothalamus, respectively. After the probe signals were normalized to the median expression levels of all probes included in the microarray, probes with signals that differed greater than twofold in intensity than those of control group were analyzed. We used the significance analysis of microarrays *t* statistic method for microarray statistical analysis [8].

We then selected probes with *t*-test *p* values <0.05 and performed Gene Ontology (GO) analysis of 1460 and 992 probes selected from the frontal cortex and hypothalamus, respectively, with levels of expression that were lower than 2-fold. Further, of 24,305 and 25,089 probes of the frontal cortex and hypothalamus, respectively, 23,455 common were merged, and ANOVA was performed to compare the data of each of the four experimental and control groups prepared from each tissue. After the *p* value calculated using ANOVA was converted to the False Discovery Rate (FDR), we performed eight divided cluster analyses of 14,248 probes with FDR values <0.05.

2.5. Effects of CO_2 concentration on mRNA levels

Of 50 male Wistar rats (8-weeks-old, 301–360 g body weight) (Kyudo Co., Ltd.), 10 were allowed to breathe air (control group), and groups of 10 each of the remainder were exposed to 10%, 20%, 40%, and 60% CO_2 gas, respectively. For each CO_2 concentration, the O_2 concentration was maintained at 20% and the N_2 concentration was adjusted according to the CO_2 concentration. After exposure, cervical dislocation was used to sacrifice the rats. Total RNA was isolated, and cDNA was synthesized as described in Section 2.2. The cDNAs were stored at −20 °C.

2.6. Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction (PCR) was performed in a 20-μl reaction mix using the TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems) with a StepOne Real Time PCR System (Applied Biosystems). The contents of the amplification mix and thermal cycling conditions were set according to the manufacturer's instructions. The primers for alkylglycerone phosphate synthase (*Agps*), hypocretin (*Hcrt*), tyrosine hydroxylase (*Th*), heat shock protein beta 2 (*Hspb2*), opioid receptor delta 1 (*Oprd1*), renin (*Ren*), pancreatic polypeptide (*Ppy*), corticotropin releasing hormone receptor 2 (*Crhr2*), carbonic anhydrase 1 (*Car1*), and hypocretin receptor 1 (*Hcrtr1*) were purchased from Applied Biosystems. The expression levels of the target transcripts are presented as the ratio of the targets normalized to those of the reference β-actin (*Actb*) mRNA.

2.7. Analysis of mRNAs expressed in the brain

The expression levels of each mRNA were determined using the ΔΔCt method. All statistical analyses were performed for the ΔCt data to exclude potential bias due to the averaging of data calculated using the equation $2^{-\Delta\Delta Ct}$. To compare data between groups, the nonparametric test Steel–Dwass test was performed using JMP11 software (SAS, Cary, NC, USA). A *p* value <0.01 was considered statistically significant.

3. Results

3.1. Selection of candidate genes

Table 1 shows the number of probes with signals that varied in intensity by more than twice the value of variation compared with those of the control group with SAM *t*-test values <0.05. The numbers of genes downregulated twofold were greater than those of the genes upregulated twofold. GO analysis of the downregulated probes representing both tissues revealed that the most frequently represented gene clusters with low *p* values were as follows: (1) genes encoding components of G protein-coupled receptor pathways, (2) genes encoding proteins involved in olfaction, and (3) genes encoding proteins involved in transcription (Table 2).

Among the probes detected in each tissue, genes encoding proteins that mediate homeostasis and nerve function are involved in components of G protein-coupled receptor pathways. Further, cluster analysis of 24,305 and 25,089 probes representing the frontal cortex and hypothalamus, respectively, revealed significant differences between the experimental and control groups in clusters 5 and 6 (Fig. 1). Cluster 5 included the same genes related to homeostasis and the nervous system that encode components of G protein-coupled receptor pathways. According to these results, we selected the target genes as follows: frontal cortex – *Agps*, *Hcrt*, *Th*, *Hspb2*, and *Oprd1*; hypothalamus – *Ren*, *Ppy*, *Crhr2*, *Car1*, and *Hcrtr1* (Table 3).

Table 1
Computation of gene expression levels.

	Frontal cortex	Hypothalamus
2 up-regulation and SAM (*t*-test) < 0.05	146/24,305	211/25,089
2 down-regulation and SAM (*t*-test) < 0.05	1460/24,305	922/25,089