of each gene and to introduce a single threshold value (=0.75) to filter the genes in which the expression levels altered. (iii) All the relative log ratios were arranged into a matrix (designated secondary data matrix). (iv) In order to filter out the genes in which the expression levels deviated from the mean average in an individual data set for each gene, each time point, and each organ, we calculated standard deviation of relative log ratios for each time point and each organ, using the data from all the animals (Exp. 1 and Exp. 2). The SD values calculated by every organ showed similar distributions and there was no obvious correlation between the SD values and the fluorescent intensities initially detected in each microarray. This enabled us to introduce a single SD value (=1.0) as a cutoff threshold for all the genes. The calculated value should reflect the differences between independently repeated anesthetic experiments and the responses of individual rats. (v) The genes with standard deviation of the relative log ratios greater than 1 for at least one time point for each organ were detected from the secondary data matrix. (vi) The genes with relative log ratios commonly greater than 0.75 or uniformly smaller than −0.75 in each organ among at least five individual rats, which had an identical anesthetic period (2 h or 6 h), were selected from the secondary data matrix.

Next, we extracted genes, in which expression patterns were specific to a single organ or common to multiple organs from the secondary data matrix by the following operations. (i) The mean average of log ratios for 2 h or 6 h for each organ (designated 2-h and 6-h averages) was calculated. (ii) The relative ratios of 2-h and 6-h averages against 0-h average for each organ (designated relative 2-h and 6-h averages) were generated. (iii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in a single organ were selected. (iv) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any two organs were selected. (v) Genes with relative 2-h average or relative 6-h average was greater than 0.75 or lower than −0.75 in any three organs were selected. (vi) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any four organs were selected. (vii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in more than four organs were selected.

3. Results

In order to comprehensively evaluate the influences of general anesthesia at the gene expression level, we obtained seven major organs from rats under general anesthesia with sevoflurane, an inhalation anesthetic, at 0 h, 2 h, and 6 h after induction of anesthesia. We performed the animal experiment with sevoflurane twice with an interval of several weeks between the two experiments (designated as Exp. 1 and Exp. 2). In Exp. 1, we used three rats for each time point and obtained for the seven organs but failed to draw peripheral blood from a rat assigned for 2 h. In Exp. 2, we used four rats for 0 h and three rats for 2 h and 6 h, respectively, and failed tissue lysate preparation from the lungs and liver of a rat assigned for 0 h. Eventually, we obtained 130 independent tissue samples for the three time points. We labeled poly(A)+ RNA purified from the samples and a rat common reference RNA with Cyanine-5 and Cyanine-3, respectively, and hybridized to microarrays representing 11,464 transcripts derived from 10,490 individual genes. Hybridization signals were processed into expression ratios as $\log_2$ values (designated log ratios) and compiled into a matrix designated as the primary data matrix (see Materials and methods). Data from all the arrays used in this paper are available at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX4 (for the array design) and CAR4 (for the experiments).

3.1. Overview of gene expression patterns in rat tissues after being subjected to general anesthesia

First, we sought to seize an overview of the experiments conducted in this study by two-dimensional clustering analysis of log ratios calculated from primary expression ratios against the common reference RNA (primary data matrix), prior to extracting genes that were affected by inhalation anesthesia at the expression level. The primary data matrix should enable us to relatively compare all samples one another without conducting direct comparison by hybridization on an identical microarray. We predicted that the most obvious differences obtained from the clustering analysis of the primary data matrix should be those reflecting tissues examined in this study, represented as sample clusters consisting of each organ only (tissue clusters) in a single dendrogram. Furthermore, we presumed that we might be able to compare gene expression levels that were associated with different experimental conditions within each tissue cluster in the dendrogram.

We extracted genes with log ratios over 1 or under −1 in at least one sample from the primary data matrix and subjected them to two-dimensional hierarchical clustering analysis for samples and genes (Fig. 1) (Schena et al., 1996; Lyons et al., 2000). When the clustering analysis for the samples was performed, the greatest seven clusters corresponding to the individual organs were obtained as predicted. The clusters representing the lungs, heart, liver, and blood comprised two smaller clusters completely corresponding to differences between Exp. 1 and Exp. 2, reflecting experimental errors. The clusters representing the kidney and brain consisted of two smaller clusters that incompletely corresponded to the experiment differences with some exceptions. With the exception of the smaller cluster in Exp. 1, representing the liver, in which three

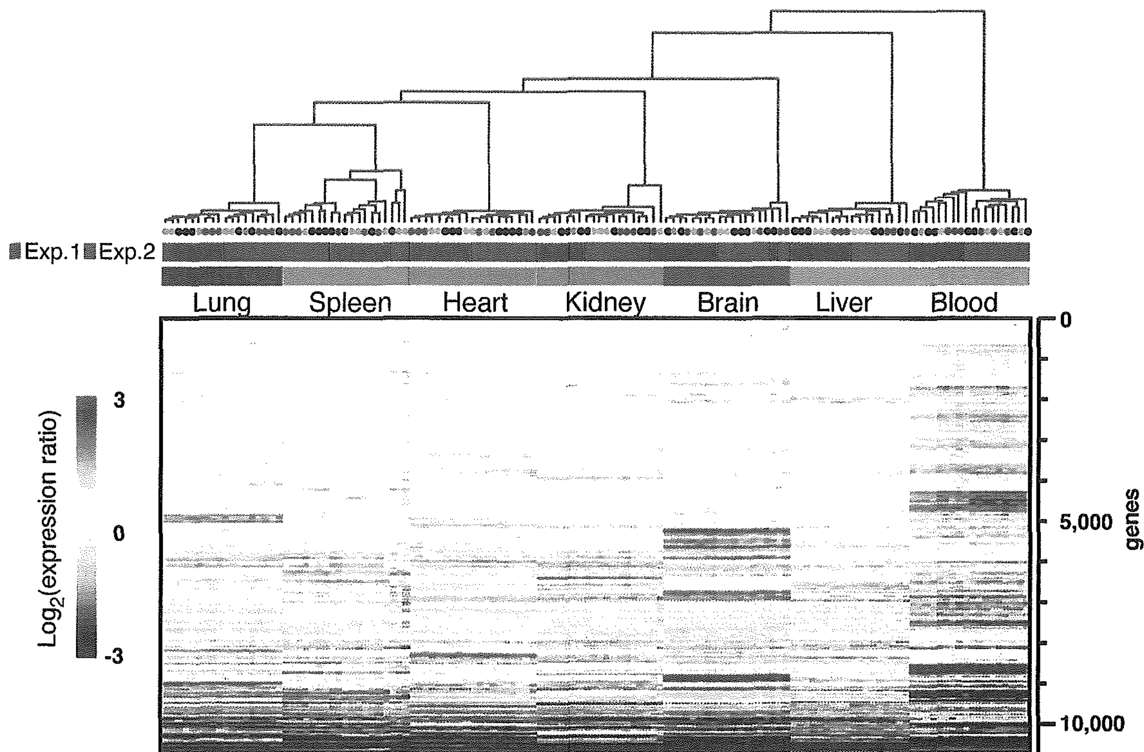


Fig. 1. Gene expression profiles obtained from seven organs of rats under inhalation anesthesia. Rows and columns represent genes (10,796) and samples (130), respectively. The genes and samples are assembled in the order obtained from the results of the two-dimensional hierarchical clustering analysis. Dendrogram at the top of the figure indicates the relationship among the samples after clustering analysis; the y-axis of the dendrogram depicts Euclid square distance as the dissimilarity coefficient. The color bar on the left side of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Solid circles indicate individual rats assigned for 0 h, 2 h, and 6 h by black, green, and red, respectively. Red and blue bars show two independent anesthesia experiments, i.e., Exp. 1 and Exp. 2, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

different time points formed the three smallest clusters, the smaller clusters by the differences in experiment were not divided by differences of anesthetic periods but by the individual differences among rats. These results obtained from the clustering analysis for the 130 samples indicate that differences among anesthesia periods with regard to the expression profiles of over 10,000 genes are much smaller than differences among individual rats, independent experiments, and organs.

3.2. Selection of genes in which expression patterns were affected by inhalation anesthesia

In order to extract genes in which expression levels altered specifically due to inhalation anesthesia, from the primary data matrix, we conducted the operations described in the Materials and methods and generated the secondary data matrix comprising relative log ratios. From the secondary data matrix, we successfully obtained 177 transcripts originating from 167 genes that satisfied the conditions described in the Materials and methods and subsequently subjected the data of the 177 transcripts to clustering analysis for genes (Fig. 2). Clustering analysis demonstrated that the 167 genes comprised those genes specific to a single organ and common in different organs. It also demonstrated that among the organs tested in this study, expression alteration of 114 genes was predominantly detected in the liver and that of 42 genes was predominantly detected in the lungs and the expression alteration of a few genes was predominantly detected in the blood, brain, heart, kidney, and spleen. Moreover, we noticed that the alteration of expression levels was detected mainly in the longer period but rarely in the shorter period. These results confirm that the inhalation anesthesia affected expression of a small number of genes except for the liver. This result was also confirmed by the two-dimensional clustering analysis for the primary data matrix of over 10,000 genes.

3.3. Expression profiles for genes that are immediately affected by anesthesia

Next, we extracted genes whose expression levels were immediately affected by the inhalation anesthesia, particularly in the shorter period by filtering operations similar to those described above. The reason was the following. Although we noticed that the predominant alteration appeared at the time point of 6 h, we expected the possibility that the data obtained at 2 h might provide information on genes that may specifically regulate the expression of the

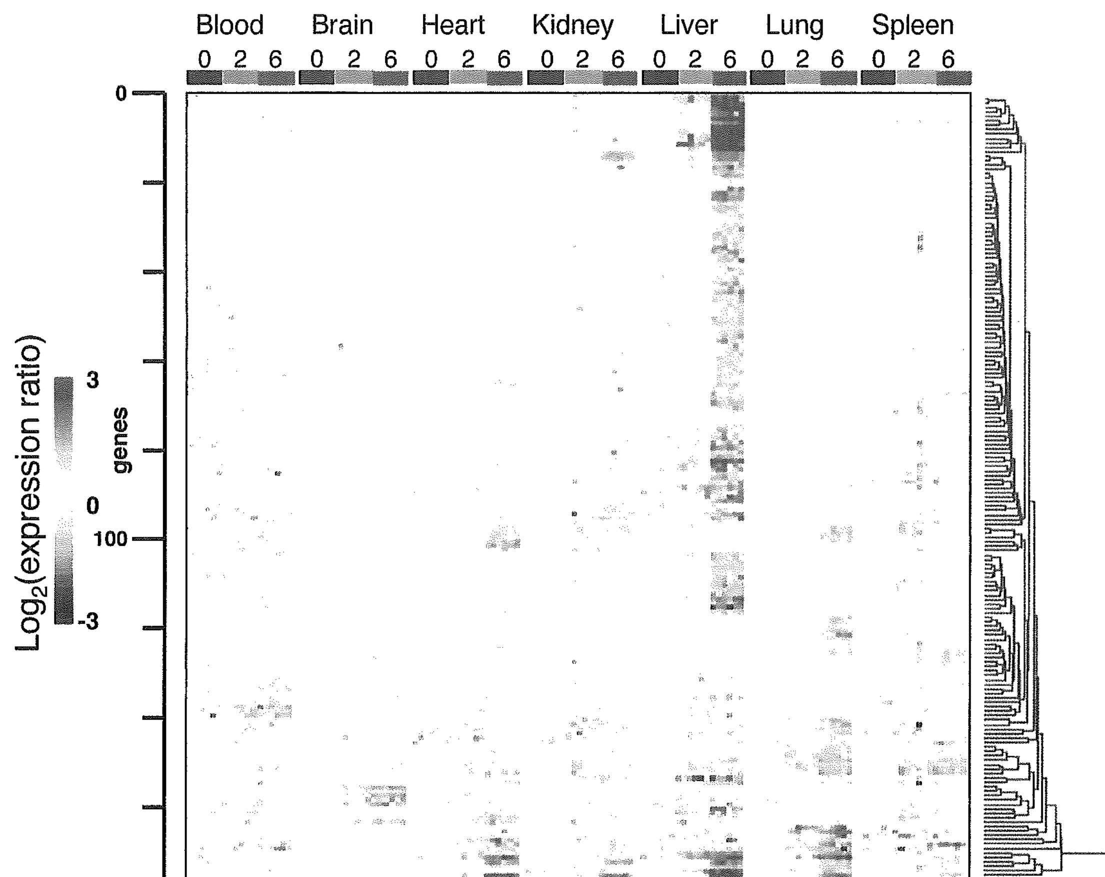


Fig. 2. Clustering analysis of genes in which expression levels were obviously altered in an inhalation anesthesia-specific manner. Rows and columns represent genes and samples, respectively. Rows are assembled in the order derived from clustering analysis. Columns are assembled in the order of organs and time points; black, green, and red bars exhibit 0-h, 2-h, and 6-h samples, respectively. Dendrogram on the right side of the figure indicates the relationship of genes after clustering. The color bar on the left side of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

genes affected in the later phase. We obtained only 20 transcripts derived from 18 genes, as genes affected immediately by the inhalation anesthesia (Fig. 3). These results indicate that in the early phase, an extremely small number of genes are affected by inhalation anesthesia (approximately 0.17% of the total genes tested). Among the 20 transcripts, 18 exhibited single organ-specific expression patterns but only two represented those common in multiple organs. One of the two genes common to the multiple organs was a gene previously known as the circadian gene, *Rev-ErbA-alpha* (Leloup and Goldbeter, 2003). We notice that the gene expressing vasoconstrictor endothelin 1 (Yanagisawa et al., 1988) was upregulated as an early responsive gene in the lungs. This may be because inhalation anesthetics are initially exposed at the highest concentration to the lungs as compared to the otherorgans.

3.4. Extraction of genes in which expression was influenced by inhalation anesthesia in an organ-specific manner

We extracted genes in which expression patterns were specific to a single organ or common in different organs by the operations described in the Materials and methods. Subsequently, we selected genes that satisfied the conditions described in the Materials and methods from the genes shown in Fig. 2. We classified the selected genes in which the expression patterns were influenced by the inhalation anesthesia, as shown in Fig. 4a–e.

In the liver, we detected the maximum number of genes in which the expression was influenced by the inhalation anesthesia (99 transcripts; approximately 56% of the total genes influenced). These liver-specific genes include those characterized as the drug metabolism-associated or the drug response-associated genes such as cytochrome *P*450s (Lu, 1998), epoxide hydrolases (Cannady et al., 2002), UDP-glucuronosyl transferases (Mackenzie et al., 1997), and glutathione sulfotransferases (Snyder and Maddison, 1997). We obtained the second highest number of influenced genes in the lungs (15 transcripts). Since anesthetics act on the brain and induce unconsciousness, we focused on the genes in which the expression specifically influenced the brain. We did not observe an elevation of expression levels of any genes in the brain but detected four genes in which expression levels decreased after the induction of the inhalation

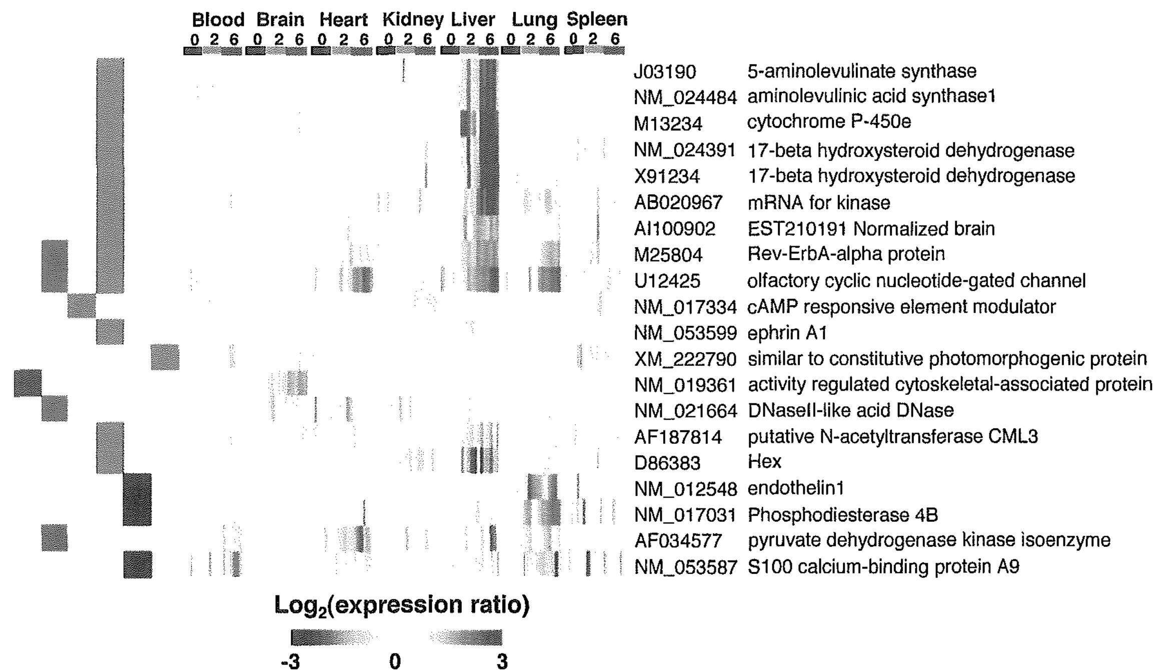


Fig. 3. Genes in which expression levels altered in the early phase (2 h) of inhalation anesthesia. The genes are assembled in the order obtained from the results of hierarchical clustering analysis. The color bar at the bottom of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

anesthesia (*Arc* (Link et al., 1995), *NGFI-B* (Maruyama et al., 1998), *Krox20* (Bhat et al., 1992), and *Egr1* (Liu et al., 1996)). These four genes were previously reported to encode transcription factors and to be those in which the expression levels were differently affected by treatment with the agents acting on the central nervous system (CNS), such as amphetamine (Gonzalez-Nicolini and McGinty, 2002), cocaine (Freeman et al., 2002), pentobarbital (Ryabinin et al., 2000), and antidepressants (Pei et al., 2003).

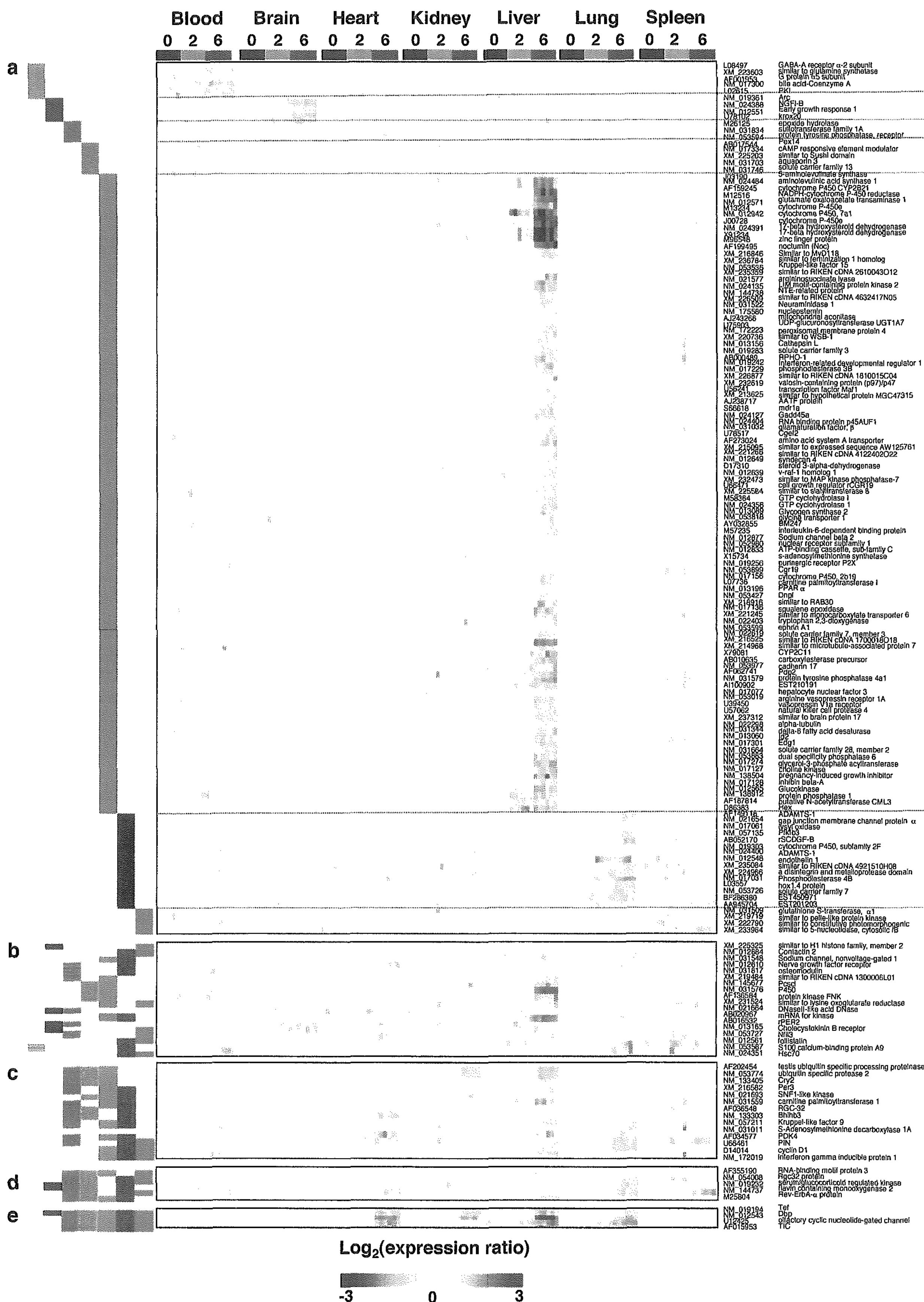
3.5. Evaluation of genes in which expression was altered during inhalation anesthesia in a multiple organ-common manner

As described above, we notice that several genes were affected during anesthesia in a similar manner among multiple organs. These included the previously reported circadian genes in which the expression level alters with an oscillating rhythm based on whether it is day or night. We selected expression data for the representative circadian genes from the secondary data matrix (Fig. 5). Most temporal expression patterns for known circadian genes obtained in this study were consistent with those reported previously with the exception of the genes from the brain (Ueda et al., 2002; Storch et al., 2002; Panda et al., 2002). In the brain, we observed no alteration of the expression of the known circadian genes during anesthesia with the exception of *Per2*. The *Per2* gene provided an expression pattern contradictory to those reported previously, representing decreased expression levels during the day. Furthermore, we compared expression patterns obtained in this study with those recently reported for the mouse circadian genes using the microarray technology (Ueda et al., 2002). The rat orthologues of the mouse circadian genes in the brain exhibited no alteration of expression during the inhalation anesthesia. The genes compared will be listed in Supplementary Information Table 6 (http://www.cibex.nig.ac.jp/cibex/HTML/index.html; under accession no. CAR4). These findings indicate that under inhalation anesthesia, the circadian rhythm may differ from normal circadian rhythms.

4. Discussion

By analyzing expression profiles obtained from rats under general anesthesia,we have shown that inhalation anesthesia affected expression of a small number of genes.

Fig. 4. Classification of genes that showed alteration in expression levels during anesthesia by the number of the organs in which the alteration was detected. a, genes in which expression levels altered in a single organ-specific manner. The genes of each gene set for a specific organ are assembled in the order derived from clustering. b, c, d, e, and f, genes in which expression levels altered commonly in two, three, four, and more than four organs, respectively. The color bar at the bottom of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.


Blood
Brain
Heart
Kidney
Liver
Lung
Spleen
0 2 6
a
b
c
d
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Log2(expression ratio)
-3
0
3

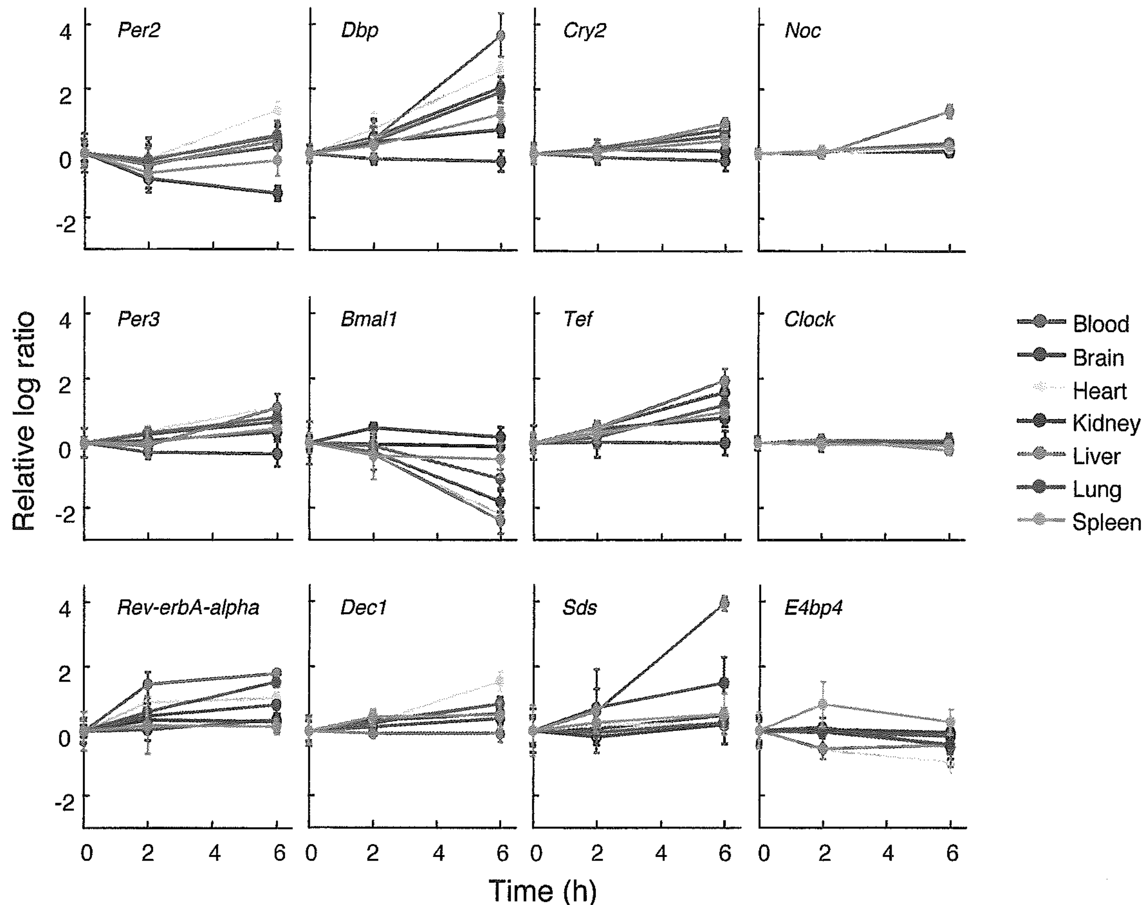

Fig. 5. Expression kinetics of the rat orthologues for the previously identified mouse circadian genes in rats under inhalation anesthesia. Relative log ratios were extracted from the secondary data matrix for the following genes: *Clock*, *Per2*, *Cry2*, *Per3*, *Noc*, *Tef*, *Rev-erbA-alpha*, *Dbp*, and *Bmal1*. The extracted relative log ratios were subjected to calculation of mean average (solid circle) and standard deviation (error bar) for each time point of individual organs. The x and y axes show time (h) and relative log ratio against the 0-h control, respectively. Graphs in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

Our study should provide the first comprehensive assessment for the influence of inhalation anesthesia with regard to experimental biology and genome science. Although we did not observe obvious alterations for the majority of genes tested, we successfully detected genes in which expression levels were influenced in an inhalation anesthesia-dependent manner.

Among the genes in which expression levels altered in an anesthesia-dependent manner, the most predominant gene cluster was found in the samples obtained from the liver. The genes will be listed in Supplementary Information Table 4 (http://www.cibex.nig.ac.jp/cibex/HTML/index.html; under accession no. CAR4). These liver-specific genes include many cytochrome *P*450s, UDP-glucuronosyl transferases, and glutathione sulfotransferases, representing involvement and activation of drug-metabolizing enzyme systems by hydroxylation, glucuronide conjugation, and glutathione conjugation, respectively (Lu, 1998; Cannady et al., 2002; Mackenzie et al., 1997; Clarke et al., 1997). This indicates that almost all the genes in the enzyme systems previously identified to be involved in drug metabolism in the liver are engaged (Gerhold et al., 2001). However, it is rather predictable that an inhalation anesthetic induces the expression of the drug metabolism-related genes as a toxicological response. On the other hand, as novel findings, our data propose that many transcripts (thus far uncharacterized) may be regulated by a common set of trasncription factors, in addition to being involved in the metabolism of the anesthetic in a similar manner to the genes for drug-metabolizing enzymes that were previously identified. Moreover, we presume that the induction of endothelin at the early phase of anesthesia in the lungs represents one of the toxicological responses and that endothelin possibly induces some effects in the vascular system, particularly in the early phase of the anesthetic period. This is presumed because the induction of the endothelin gene expression has been previously reported as responses to toxins (Sonin et al., 1999; Baveja et al., 2002). It needs to be investigated whether other anesthetics affect the expression of the genes in a manner similar to sevoflurane, as demonstrated in this study. Such a comparative study would provide information that is necessary to assess the side effects of the currently available anesthetics and to develop safe tools in anesthesiology.

We should mention that the findings presented here may not provide direct evidence to suggest that the anesthetic causes the anesthetic status by inducing alteration in the expression of certain genes since we did not detect many regulated genes in the brain, which should comprise the main target of the anesthetic. Despite the low number of regulated genes (3 up, 6 down), these genes encode transcription factors and circadian rhythm genes that were previously reported to be increased by amphetamine (Gonzalez-Nicolini and McGinty, 2002), cocaine (Freeman et al., 2002), and morphine withdrawal (Ammon et al., 2003), whereas their expression decreases by anesthesia with an intravenous anesthetic (Ryabinin et al., 2000).

One of the interesting findings of this study is that several circadian genes exhibited alteration of expression during an anesthetic period in a multiple organ-common manner. However, in the brain, we obtained several findings that were inconsistent with those reported previously (Ueda et al., 2002). We did not detect an alteration in several genes in which expression exhibited the circadian rhythm and we observed contradictory kinetics in the expression of *Per2*. The hypothesis that inhalation anesthesia perturbs the circadian rhythms requires further investigations. We assume that one of the reasons for these differences may be due to the tissue used for analysis. Most previous studies focused on the center of the circadian rhythm, the suprachiasmatic nucleus (Ueda et al., 2002; Panda et al., 2002). On the other hand, we used the whole brain as a target. The suprachiasmatic nucleus occupies an extremely small region of the whole brain. Therefore, the dilution of mRNA of the suprachiasmatic nucleus with mRNA of the whole brain might have affected the detection of the expression of the circadian genes mainly regulated in the suprachiasmatic nucleus. Furthermore, to obtain conclusive evidence for the association of anesthetic periods and the expression of circadian genes, future studies should scrutinize expression profiles for circadian genes at more multiple time points with a shorter interval than those used in this study. In order to further analyze the circadian gene expression, it would be useful to perform a non-microarray approach such as real-time PCR for the limited number of target genes as shown in this study.

In this study, we independently repeated the identical anesthetic experiment twice with rats. Clustering analysis shown in Fig. 1 exhibits that with the exception of the brain and kidney, in the other five organs smaller clusters are generated for each, which correspond to the two independent anesthetic experiments, reflecting experimental errors. Only the brain and kidney do not exhibit such smaller clusters that completely corresponded to the two independent experiments, indicating that in these two organs, experimental errors between the two experiments were smaller than the differences among individual rats and/or experimental conditions for anesthesia. These results clearly indicate that experimental errors among independent anesthetic experiments are not negligible and that one should be careful while drawing conclusions from a single experiment with animals under mildly different experimental conditions such as inhalation anesthesia. We successfully avoided highlighting genes that reflected the experimental errors by repeating the identical anesthetic experiments and conducting filtering operations for a combined gene expression data set. These findings provide experimental evidence that endorses the significance of repeating an independent experiment with animals under experimental conditions with mild differences and of data processing to reduce noises that reflect the differences among the repeated experiments.

As described above, our study demonstrates that the experimental errors observed between the independently repeated anesthetic experiments provided the second highest difference that follows the differences among the individual organs tested, which generated the most distinguishable clusters. This finding led us to use threshold-based approaches with strict cutoff values to extract genes in which expression was specifically affected by inhalation anesthesia and to avoid influence of the experimental errors and the differences among individual rats assigned for the identical conditions. These relatively strict analytical approaches may result in insufficient extraction of specifically affected genes, particularly for those in which expression was altered in a subtle manner. One of the analytical approaches that may enable us to obtain a greater number of genes specifically affected by inhalation anesthesia should be to apply lower thresholds in the filtering operation described in the Materials and methods for the data set compiled in this study. The approach may be effective to obtain as much information as possible from our primary data set. However, this would require additional confirmation for the individual values through non-microarray approaches.

We have provided a large and powerful data set for exploring the influence of inhalation anesthesia in rats. The entire data set will be available in a public database. The results presented here were derived from the data set by relatively conservative approaches due to the experimental differences greater than those expected prior to data analysis. Therefore, apart from conducting additional more fine-tuned experiments, another approach may be helpful for the further understanding of comprehensive influence of anesthesia by means of utilizing advanced statistical methods such as analysis of variance (ANOVA). Application of the more sophisticated approaches would enable those who use the data set to explore additional categories of genes that are modulated under anesthesia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.03.022.

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Original Article

Anaesthesia and circulating blood volume

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Summary

Background and objective: The exact change in circulating blood volume (BV) during general anaesthesia is still unknown because there is no standard method of evaluating BV. We evaluated the changes in BV by general anaesthesia using simple and easy estimation methods.

Methods: Fourteen patients scheduled for minor surgery under general anaesthesia were enrolled. Propofol and vecuronium bromide were used for the induction of anaesthesia, and anaesthesia was maintained with sevoflurane and nitrous oxide. Haematocrit (Hct), total protein concentration (TP), as well as colloid osmotic pressure (COP) measured using a colloid osmometer, were determined before anaesthesia, and 30, 60 and 90 min after the induction of general anaesthesia. BV was calculated using Allen's formula and the changes in Hct, TP and COP. The estimated BV was compared with directly measured BV using indocyanine green dilution method (BV_{ICG}).

Results: Hct, TP and COP significantly decreased after the induction of anaesthesia (Hct: 42.1–39.4%; TP: 7.3–6.9 g dL^{-1}; COP: 23–19 mmHg). The calculated BV as well as BV_{ICG} significantly increased after induction of anaesthesia (calculated by COP: 4.13–5.03 L; BV_{ICG}: 4.54–5.56 L). The change rate in BV calculated by the change of COP was larger than other calculated BVs, and was approximated to the change rate in BV_{ICG}. After emergence from anaesthesia, all values tended to return to baseline.

Conclusions: General anaesthesia increases BV. The value of BV calculated from the change in COP was most changeable.

Keywords: GENERAL ANAESTHESIA; CIRCULATING BLOOD VOLUME; COLLOID OSMOTIC PRESSURE.

It is well known that general anaesthesia decreases blood pressure (BP) through vasodilatory as well as negative inotropic and chronotropic effects. Vasodilatation results in insufficient circulating blood volume (BV) to maintain BP (relative hypovolaemia). However, few reports have observed the actual changes in BV induced by general anaesthesia [1–3].

The standard method for measuring BV has been indicator dilution, using either a radioisotope or dye [4–8]. It is difficult to measure BV serially with these methods because these tracers are retained in the blood for days [9–11]. Furthermore, minor risks associated with the use of radioactive iodine and mutagenicity of Evans blue dye have been reported [12–16].

In 1956, Fox and colleagues [17] introduced indocyanine green (ICG) dye, which is now the pulse indicator dye of choice for determining BV. ICG has no known side-effects, other than a rare iodine-induced allergic reaction. However, this method requires a long interval between each measurement point, and cannot be used in patients with hepatic failure. Therefore, we used changes in haematocrit (Hct), total

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serum protein concentration (TP), and plasma colloid osmotic pressure (COP) in patients undergoing minor surgery to estimate the BV changes induced by general anaesthesia.

Methods

Fourteen patients undergoing minor surgery were included in this study. All patients were ASA I, and had no history of cardiovascular, pulmonary, or neurological disorders, arteriosclerosis or allergies. No patients had taken medication during the 2 weeks prior to the study. The study protocol was approved by the Ethics Committee of the Nippon Medical School, and written informed consent was obtained from all patients the day before surgery.

All patients fasted from 9 o'clock the night before surgery, and no patient was premedicated or given any intravenous (i.v.) fluid before the induction of anaesthesia. When the patients arrived in the operating theatre, a catheter was placed into the dorsalis pedis artery because the upper body was draped during the procedure. Blood samples were obtained through this line during the study period. Before anaesthesia, systolic blood pressure (SBP), Hct, TP and COP were measured and defined as the baseline values ('Awake'), and estimated BV value of each patient was calculated from the formula (BV_{Allen}) of Allen and colleagues [18].

$$\text{Equation for male: } 0.417 \times \text{height}^3\ (\text{m}) + 0.045 \times \text{body weight (kg)} - 0.03\ (\text{L}).$$

$$\text{Equation for female: } 0.414 \times \text{height}^3\ (\text{m}) + 0.0328 \times \text{body weight (kg)} - 0.03\ (\text{L}).$$

A peripheral i.v. cannula (18-G) was inserted under local anaesthesia, and a solution of 5% glucose was administered using an infusion pump at a rate of $2\,mL\,kg^{-1}\,h^{-1}$ throughout the study period. The choice of a 5% glucose solution was made to prevent dehydration, while having less of an effect, and to affect on BV than colloid or electrolyte solutions. SBP was monitored via an automated non-invasive sphygmomanometer every 5 min. Other monitors included an electrocardiogram, pulse oxymeter and capnometer. Hct and TP were measured using standard techniques. COP was measured with a colloid osmometer (Wescor-4420; Wescor Inc, Logan, UT, USA). In brief, the operating principle of this colloid osmometer is based upon the movement of water molecules and diffusible solute particles through a synthetic semi-permeable membrane which has a diffusion cut-off of 30 000 Da the phenomenon known as transudation [19]. The membrane separates the specimen solution from a reference solution. After a sample is injected to the reference chamber, fluid moves through the membrane and into the sample chamber until the hydrostatic pressure reaches equilibrium. This pressure is measured by a piezoelectric pressure transducer.

General anaesthesia was induced in all patients with propofol ($2.0\,mg\,kg^{-1}$) and vecuronium bromide ($0.15\,mg\,kg^{-1}$) as a rapid i.v. bolus injection to facilitate endotracheal intubation using 5% glucose (5 mL) to wash through the induction agents. Intubation was performed about 5 min after induction, and an HME filter (heat-moisture exchanger; Nercore Hygroback S filter, Nercore Puritan Bennett Inc., Pleasanton, CA, USA) was connected to the endotracheal tube. The patient was mechanically ventilated with a tidal volume of $10–12\,mL\,kg^{-1}$ at a respiratory rate of 8–10 breaths min^{-1} to maintain an end-tidal CO_2 of 35–40 mmHg during the anaesthetic period. Anaesthesia was maintained with sevoflurane supplemented with nitrous oxide (67%) in oxygen. The sevoflurane concentration was controlled to maintain the SBP to within 20% change of the 'Awake' SBP. A urethral catheter was inserted immediately after induction for urine collection. The blood loss was calculated by weighing the surgical sponges every 30 min during the study period. SBP, Hct, TP and COP were measured 30, 60 and 90 min after the induction of anaesthesia ('Anaesth-30', '-60' and '-90', respectively). At the end of surgery, the sevoflurane was discontinued, and atropine (1.0 mg) and neostigmine (2.5 mg) were injected to reverse the effect of vecuronium. The patients were left undisturbed breathing supplemental oxygen ($6\,L\,min^{-1}$) administered via face mask. The last measurement was performed 30 min after extubation ('Recovery').

BV at each sampling time was estimated from the baseline BV_{Allen} and the subsequent changes of Hct, TP and COP, respectively. For example, BV estimated from the change in Hct at 'Anaesth-30' was calculated by the following formula:

$$\text{BV (Anaesth-30: Hct)} = BV_{Allen}\ (\text{Awake}) \times [\text{Hct (Awake)}/\text{Hct (Anaesth-30)}].$$

Furthermore, BV measurement using the ICG dilution method (BV_{ICG}) was performed at 'Awake', 'Anaesth-30', 'Anaesth-60', 'Anaesth-90' and 'Awake' to compare with the calculated BV values mentioned above. A finger probe, which is connected to integrated pulse-spectrophotometry monitoring system (DDG 1001; Nihon Kohden, Tokyo, Japan) was applied to the left index finger to detect the blood concentration of ICG based on pulse spectrophotometry [20]. After the blood sampling in each time, 20 mg of ICG with 5% glucose (5 mL) was administered i.v.

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All data are expressed as the mean ± standard deviation (SD) unless otherwise stated. Correlation between BV values calculated from Allen's formula and BV values estimated using BV_{ICG} was analysed by the Pearson correlative coefficient test. The agreement between two BV measurements was assessed by Bland–Altman analysis. A two-way analysis of variance (repeated-measures ANOVA) was performed comparing measured and calculated variables at each time point. A P value <0.05 was considered significant. When a significant difference was found, Tukey's multiple comparison test was performed to compare other values with the 'Awake' value.

Results

Table 1 summarizes the clinical characteristics of the patients in this study. The significant difference arising from sex difference was seen with the mean BV_{Allen} values at 'Awake' (F : M, 3.12 : 5.49 L, respectively). Types of surgery included tympanoplasty ($n = 3$), sinus surgery ($n = 6$), parotidectomy ($n = 4$) and skin graft ($n = 1$).

The changes in SBP, Hct, TP, COP and BV_{ICG} during the study are summarized in Table 2. The significant difference arising from sex difference was seen only with the Hct values, however, the ratios of changes in Hct did not differ between female and male. The mean SBP was decreased at 'Anaesth-30', and remained stable thereafter. At the 'Recovery' time point, the SBP was recovered to the 'Awake' value. The Hct, TP, COP and BV_{ICG} were lower at 'Anaesth-30', and remained stable during anaesthesia. Only the COP was not returned to the 'Awake' value by the 'Recovery' time point.

The BV_{Allen} and BV_{ICG} at 'Awake' in 14 patients had a significant correlation ($r^2 = 0.775$; $P < 0.01$). Bland-Altman analysis resulted in a bias of −0.195 L with limits of agreement from −1.309 L to 1.061 L (Fig. 1). The BV changes by four kinds of methods during the study were shown in Figure 2. The mean increase rates of BV_{ICG} and BV estimated by COP were higher than those estimated by the other markers at Anaesth-30, Anaesth-60 and Anaesth-90.

Table 1. Study patients' data and clinical characteristics of undergoing surgery.

Number	14
Gender (M : F)	7 : 7
Age (yr)	43.8 ± 13.7 (27–57)
Height (cm)	163 ± 11 (151–181)
Weight (kg)	59.3 ± 13.6 (43–80)
Time of anaesthesia (min)	181 ± 45 (95–230)
Blood loss (mL)	63 ± 71 (0–230)
Urine volume ($mL\,h^{-1}$)	48 ± 56 (10–69)
BV_{Allen} at 'Awake' (L)	4.13 ± 1.23 (2.31–6.69)

Data are presented as the mean ± SD (range). BV_{Allen} at 'Awake', estimated circulating BV calculated form Allen's formula before induction of anaesthesia.

Discussion

Fluid is infused during surgery to correct dehydration due to preoperative fasting, transpiration, metabolism and blood loss to maintain the BV. Vasodilatation caused by general anaesthesia results in a relative decrease in BV and requires a larger infusion volume to maintain tissue perfusion.

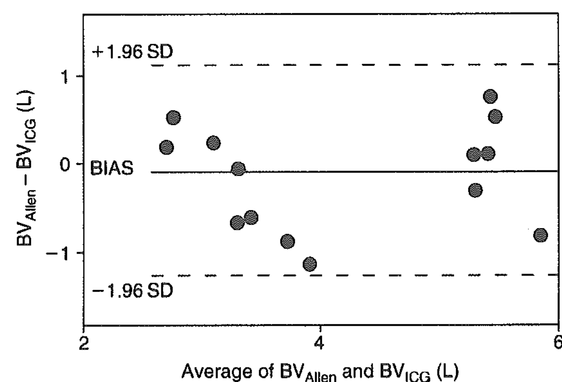

Figure 1.
Bland–Altman bias plot for the comparison of the blood volume calculated from Allen's formula and the blood volume estimated by the BV_{ICG}. BV_{Allen}: blood volume calculated from Allen's formula; BV_{ICG}: blood volume estimated by the BV_{ICG}. The dotted line indicates 1.96 SD around the mean difference (bias; fine line) (n = *14).*

Table 2. Sequential changes in the SBP, Hct, TP, COP and BV_{ICG}.

	Awake	Anaesth-30	Anaesth-60	Anaesth-90	Recovery
SBP (mmHg)	111 ± 20	93 ± 13*	93 ± 15*	92 ± 10*	117 ± 15
Hct (%)	41.9 ± 4.6	39.2 ± 4.5*	38.2 ± 5.0*	38.4 ± 5.0*	40.4 ± 4.7
TP ($g\,dL^{-1}$)	7.3 ± 0.6	6.9 ± 0.5	6.6 ± 0.5*	6.7 ± 0.5*	7.0 ± 0.6
COP (mmHg)	23.0 ± 4.7	19.0 ± 4.4*	18.4 ± 3.3*	18.3 ± 3.8*	20.3 ± 4.2*
BV_{ICG} (L)	4.54 ± 0.80	5.56 ± 1.24*	5.56 ± 1.65*	5.60 ± 1.53*	4.58 ± 1.13

Data are presented as the mean ± SD ($n = 14$, each). *$P < 0.05$ compared to the 'Awake' value.

Determining the effect of general anaesthesia on BV is an essential component of perioperative fluid management [21]. However, no consensus exists as to how to measure the BV accurately intraoperatively.

Allen's equation has been used as a basic model for normovolaemic haemodilution, and many studies used this equation as an index of baseline blood volume [10,22]. In 1989, Hahn [11] gave similar results to measurement of the blood volume with the ^{131}I-RISA technique in 10 patients scheduled for transurethral resection of prostate. In our study, BV_{ICG} in 14 patients was significantly correlated with that calculated from Allen's formula before induction of anaesthesia, although the confidence interval (CI) was wide which might reflect the small sample size.

The major finding of the present study is that the Hct, TP and COP decrease during general anaesthesia. Given the nature of the surgical procedures, there was probably little change in BV due to transpiration or blood loss. Furthermore, it is unlikely that appreciable numbers of erythrocytes or amounts of albumin, which determines the COP, leaked out of the intravascular space. Therefore, the continuous measurement of changes in Hct, TP and COP is an easy and reliable method for estimating the BV based on the dilution or concentration of the blood.

Plasma COP is an important determinate blood volume [23–25]. Furthermore, a low COP can cause pulmonary oedema [26]. Although changes in COP have been studied under different conditions, the changes induced by anaesthesia itself have not been studied. In this study, the COP decreased during

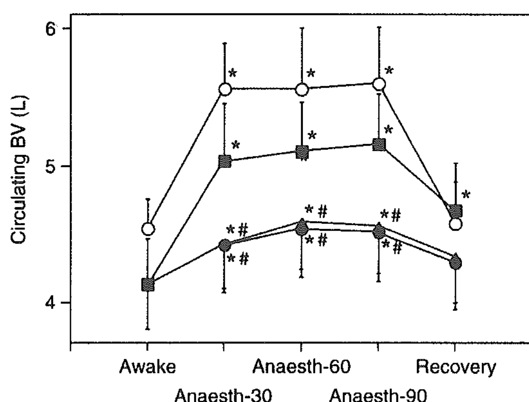

Figure 2.
Changes of the mean BV during the study period estimated by four different kinds of methods. ●: *BV estimated by Allen's formula* (BV_{Allen}) *and changes in Hct;* ▲: *BV estimated by* BV_{Allen} *and changes in TP;* ■: *BV estimated by* BV_{Allen} *and changes in COP;* ○: *BV estimated by the* BV_{ICG}. *Values are mean ± standard error (SE)* (n = 14, *each*). $^{*}P < 0.05$ *compared to 'Awake' value.* $^{\#}P < 0.05$ *compared to* BV_{ICG}.

anaesthesia. The mechanism responsible for this change is not well understood. However, Starling forces causing capillary exchange are thought to play an important role [18,27–29]. Red cell and colloid molecules are sufficiently large that they normally do not cross capillary membranes. Therefore, under normal conditions, most administered colloid remains in the intravascular space. The distribution of fluid throughout the body is dependent on the forces represented by the Starling equation:

$$J_v = K[(P_{MV} - P_T) - d(COP_{MV} - COP_T)],$$

in which J_v represents the rate of filtration of fluid across the capillary; K is the ultrafiltration coefficient (a measure of permeability); P_{MV} is the hydrostatic pressure within the capillary; P_T is the hydrostatic pressure in the interstitial space; d is the reflection coefficient representing the ability of a semipermeable membrane to prevent movement of a given solute; COP_{MV} is the COP in the capillary; and COP_T is the COP in the tissue.

The induction of anaesthesia causes a decrease in the arterial and capillary pressures. As a result, less fluid diffuses through the capillary membranes into the intravascular space, and the BV increases. Hct, TP and COP decreased during anaesthesia, but returned to baseline after anaesthesia. It is thought that the forces determining fluid exchange gradually recover to their pre-anaesthetic state. The BV value calculated from the COP increased more than that calculated from the Hct and TP. We are unable to account for this phenomenon, because we do not know what changes in the production and consumption of osmotically active colloids occurred during surgery, nor what the changes in erythrocyte size accrue as a result of osmotic changes. Further study is necessary to resolve these issues.

In conclusion, we found that the Hct, TP and COP all decreased during general anaesthesia in patients undergoing minor surgery and remained depressed throughout the anaesthesia period. Both BVs calculated by the above three kinds of materials and BV_{ICG} increased during general anaesthesia. The value of BV calculated from the change in COP was most changeable.

These results indicate that the absolute BV increases during general anaesthesia.

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—Original—

Does Carboxy-hemoglobin Serve as a Stress-induced Inflammatory Marker Reflecting Surgical Insults?

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Abstract

Endogenous carbon monoxide (CO) production has been recently observed to be an index of the inflammatory response, reflecting various insults in critically ill patients. Major surgery is supposed to modulate the production of CO by transcriptional regulation of heme oxygenase (HO). CO is easy to measure as carboxyhemoglobin (CO-Hb) by spectrophotometry; however, whether CO-Hb can be used as an index reflecting surgical insults is unknown. We investigated changes in CO generation during coronary artery bypass graft by measuring CO-Hb concentrations and the expression of HO in circulating blood as well as the expressions of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The expression ratios of heme oxygenase-1 (HO-1), TNF-α, and IL-1β significantly increased after surgery, and these values correlated significantly with one another. CO-Hb concentrations significantly increased after surgery; however, many of those values during artificial ventilation with high inspired oxygen fraction were within normal limits. Furthermore, changes in CO-Hb concentrations were small when preoperative values were high. On the whole, CO-Hb concentrations significantly but weakly correlated with the expression ratios of the inflammatory mediators. However, they did not correlate in the patients who showed higher preoperative CO-Hb concentrations. These data indicate that CO-Hb concentrations can, in general, reflect the inflammatory response induced by surgical insult; however, CO-Hb measurement may not be a useful form of clinical monitoring because of the limited degree of changes, the variation of baseline values, and the necessity for the management under fixed conditions.

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Key words: carbon monoxide, carboxyhemoglobin, heme oxygenase, tumor necrosis factor-α, interleukin-1β, surgical stress, reverse transcription polymerase chain reaction

Introduction

Major surgery is associated with the development of a systemic inflammatory response. Systemic inflammation can be potentially damaging to major organs and contributes to an increase in postoperative complications[1,2]. Although several anti-inflammatory strategies aimed at attenuating the development of a systemic inflammatory response have been used in recent years[3,4], this phenomenon varies by clinical setting. Changes in the inflammatory response can be detected by measuring plasma concentrations of certain

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inflammatory markers such as complement components, cytokines, and adhesion molecules[5,6]. However, these markers take time to measure and cannot be measured easily in all clinical settings. Thus a simple and accurate index is needed to detect perioperative changes in the inflammatory response.

In recent years, the endogenous generation of carbon monoxide (CO) has been observed to be an index of the inflammatory response after various insults[7-11]. Endogenous CO is mainly synthesized from inducible heme oxygenase-1 (HO-1) and constitutive heme oxygenase-2 (HO-2)[12]. HO-1 is a known stress-inducible heat shock protein 32 that is transcriptionally upregulated by various stressors[13]; in addition, HO-1 is inducible by more diverse stimuli than any other enzyme described to date[12]. Because it is difficult to metabolize CO in vivo, and CO binds with high affinity to hemoglobin[14], it is easy to measure the endogenous production of CO as carboxyhemoglobin (CO-Hb) using spectrophotometry.

Several clinical observations have been published on endogenous CO production in patients. In a study involving 32 surgical intensive care patients, a correlation was found between mean CO-Hb levels as high as 1.9% and severity of illness assessed by the APACHE II score[7]. Another study reported higher CO-Hb levels in 59 intensive care patients compared with 20 control patients[15]. These studies point to the possibility of using CO-Hb levels to determine a patient's severity of illness. On the other hand, another study[16] reported that plasma lactate levels and CO-Hb levels were not correlated in 183 critically ill patients and concluded that CO-Hb levels were not clinically useful as a marker of critical illness. However, previous studies did not measure the gene expression of HO that produces CO and did not compare changes in CO-Hb levels with inflammatory mediators; thus, whether CO-Hb levels are an effective index to measure surgical insults is still unknown.

In this study, we measured CO-Hb levels during elective coronary artery bypass graft surgery (CABG) in patients who underwent cardiopulmonary bypass (CPB) with or without glucocorticoid administration and in patients who did not undergo CPB. Expressions of HO-1 as well as inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in circulating blood were also measured. We evaluated 1) whether CO-Hb levels are related to HO-1 activity as measured by expression of HO-1 mRNA in circulating leukocytes, 2) whether expression of HO-1 mRNA in circulating leukocytes is elevated in surgical insults, 3) whether reduction in other markers of the inflammatory response decrease expression of HO-1 mRNA.

Subjects and Methods

The protocol of this study was approved by the review board of Nippon Medical School, and written informed consent was obtained from all subjects before the study. Patients undergoing elective isolated CABG in Nippon Medical School were included in the study. Patients undergoing re-operation, with preoperative inflammatory disease including infectious disease, perioperative use of intra-aortic balloon pumping or hemodialysis, malignant neoplastic disease, chronic or acute pulmonary dysfunction, smoking history for the past one month, or the preoperative use of steroids were excluded. No patients received anti-inflammatory drugs such as aprotinin and ulinastatin perioperatively, and all operations were performed by a single surgeon.

Preliminary Study

To evaluate the degree of change in CO-Hb concentration during CABG, arterial blood was sampled from the patients who underwent CABG during the period from August 2002 to June 2003. Although these patients fulfilled the criteria, neither the operation method nor the anesthesia methods were controlled. Arterial blood samples for serial determination of CO-Hb concentrations were taken at three timepoints: after induction of anesthesia but before sternotomy (preoperative measurement), after all bypass graftings and anticoagulation was reversed (postbypass measurement), and just before leaving the operating room (postoperative measurement). CO-Hb concentrations were

measured immediately by spectrophotometer (ABL625 blood gas analyzer, Radiometer, Copenhagen, Denmark) along with a routine blood count and blood gas analysis. The patients were divided into two groups: patients who showed CO-Hb concentrations 1% or more, and patients who showed less than 1%, and the degrees of change in CO-Hb concentration were compared.

Main Study

Following the end of the preliminary study, 45 patients participated in the main study. Patients were divided into 3 groups: (1) the on-pump group consisted of patients who underwent CPB but who did not receive glucocorticoid therapy; (2) the steroid group consisted of patients who received glucocorticoid therapy during CPB; and (3) the off-pump group consisted of patients who did not undergo CPB. Whether CPB was used for CABG was determined by each attending cardiac surgeon. Whether methylprednisolone was administered during CABG was randomly assigned. To measure gene expressions of inflammatory mediators such as HO-1, HO-2, TNF-α, and IL-1β, 4 mL of arterial blood was collected at two time points: the preoperative and postoperative periods. The sample was immediately mixed with a nucleic acid extraction reagent (ISOGEN-LS, Nippon Gene Co.,Tokyo, Japan) and stored at −70℃ until measurements were made. Arterial blood samples for determination of CO-Hb concentrations were taken at the same two timepoints. The relationships among changes in expression ratios of inflammatory mediators and the change in CO-Hb concentration were evaluated. The patients were divided into two groups: patients who showed CO-Hb concentrations 1% or more, and patients who showed less than 1%, and the degrees of correlations among CO-Hb concentration and expression ratios of inflammatory mediators were compared.

Operative and Anesthetic Procedure

Anesthesia was induced with 0.1 to 0.2 mg/kg midazolam and 2 to 4 μg/kg fentanyl and maintained with fentanyl and sevoflurane with 50% oxygen in air. Muscle relaxation was obtained with vecuronium. Other than during CPB, red cells were transfused to achieve a hemoglobin concentration greater than 10 g/dL. Surgery in all patients was performed thorough a median sternotomy. In the on-pump group, a single two-stage venous drainage cannula in the right atrium and a standard arterial cannula in the ascending aorta were used for CPB with a membrane oxygenator (HPO-2 OH-C; Senkou Ikakougyou, Tokyo, Japan). Two hundred IU/kg of heparin were administered to achieve an activated coagulation time>450 seconds. CPB prime consisted of lactate Ringer's solution and 5% glucose solution at a ratio of 4 : 1. Five mL/kg of mannitol, 1 mEq/kg of sodium bicarbonate, and 40 mL of 25% human

Table 1 Sequences of primers and probes

Heme oxygenase type 1	
	Forward primer: 5'-GGCCAGCAACAAAGTGCAA-3'
	Reverse primer: 5'-ACTGTCGCCACCAGAAAGCT-3'
	TaqMan probe: 5'-CTCCCAGGCTCCGCTTCTCCG-3'
Heme oxygenase type 2	
	Forward primer: 5'-CAGCCTTTGCCCCTTTGTACT-3'
	Reverse primer: 5'-CAAAGAAATACTCCATGTCCTTGGT-3'
	TaqMan probe: 5'-CCCCATGGAGCTGCACCGGA-3'
Tumor necrosis factor-α	
	Forward primer: 5'-ATGTTGTAGCAAACCCTCAAGCT-3'
	Reverse primer: 5'-GATGAGGTACAGGCCCTCTGAT-3'
	TaqMan probe: 5'-CTCCAGTGGCTGAACCGCCGG-3'
Interleukin-1β	
	Forward primer: 5'-AGGCTTATGTGCACGATGCA-3'
	Reverse primer: 5'-TGGACCAGACATCACCAAGCT-3'
	TaqMan probe: 5'-TACGATCACTGAACTGCACGCTCCG-3'

albumin were added to the priming solution. CPB was conducted with nonpulsatile flow at 2.2 L/min/m² with normothermia. In the steroid group, 20 mg/kg of methylprednisolone was administered just before the start of CPB. In the off-pump group, after median sternotomy, revascularization was performed on the beating and normothermic heart. Anticoagulation was achieved with heparin at 150 IU/kg after harvesting all grafts.

Real-time Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 4 mL of arterial blood using the chaotrophic Trizol method followed by Isogen-chloroform extraction and isopropanol precipitation[17]. One microgram of each total RNA extract was reverse transcribed at 37℃ for 1 h, in a 20-μL reaction mixture containing mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Takara Bio, Ohtsu, Japan). PCR primers and TaqMan fluorogenic probes were designed using the Primer Express software program (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences are shown in **Table 1**. One microliter of cDNA was used for quantitative PCR in a 50-μL volume including the TaqMan Universal Master Mix (Applied Biosystems; 25 μL); 900 nM of forward and reverse primers; 200 nM of TaqMan probe; and deionized water. PCR conditions were as follows: 50℃ for 2 min and 95℃ for 10 min followed by 40 cycles of amplification for 15 seconds at 95℃ and 1 min at 60℃. The TaqMan probe labeled with 6-FAM was cleaved during amplification, generating a fluorescent signal. Unknown samples and calibration curve samples were run in triplicate. A similar system using a separate glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and primer set (TaqMan GAPDH control reagent kit; Applied Biosystems) was designed and run for GAPDH along with every unknown sample to correct for total nucleic acid content. The assay used an instrument capable of measuring fluorescence in real time (ABI PRISM 5700 Sequence Detector; Applied Biosystems). Results of the real time PCR data were represented as the threshold cycle (CT) values, where CT was a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Relative amounts of all mRNAs were calculated by the comparative CT method (Applied Biosystems)[18] using the equation $2^{-\Delta\Delta C_T}$. ΔCT was the difference in the CT values derived from the unknown sample and the GAPDH control, while ΔΔCT represented the difference between the paired samples, as calculated by the formula ΔΔCT = ΔCT of sample before surgery − ΔCT of sample after surgery.

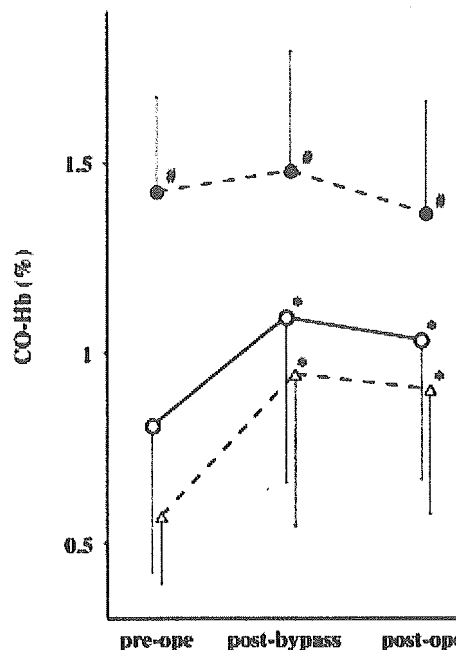

Fig. 1 Changes in the mean values of carboxy-hemoglobin (CO-Hb) concentrations in 118 patients. Open circles = mean − SD of all patients; open triangles = mean − SD of patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement (n = 85); closed circles = mean + SD of patients who showed CO-Hb concentrations ≥ 1.0% at the preoperative period measurement (n = 33). *$P < 0.05$ vs the preoperative measurement; #$P < 0.05$ vs the patients who showed CO-Hb concentrations < 1.0%.

Statistical Analysis

All values are expressed as mean ± SD. Statistical significance of the results for gene expression was

Table 2 Demographics and surgical characteristics of the 45 patients in whom the gene expressions of inflammatory mediators were measured

Group n	On-pump (n = 15)	Steroid (n = 15)	Off-pump (n = 15)	Stasistics
Gender (female/male)	4/11	5/10	5/10	NS
Age (years)	62.6 ± 6.8	63.1 ± 6.4	69.0 ± 8.8	NS
Weight (kg)	58.1 ± 15.9	59.6 ± 11.2	56.4 ± 9.4	NS
Basic diseases				
UA	2	2	1	NS
AP	7	8	9	NS
OMI	3	2	1	NS
AMI	3	3	4	NS
Risk factors				
Smoking history	8	7	7	NS
Hypertension	11	11	12	NS
Hyperlipidemia	8	8	6	NS
Diabetes mellitus	7	8	5	NS
Anesthesia time (min)	354 ± 59	365 ± 71	302 ± 68	NS
CPB time (min)	127 ± 35	132 ± 37	—	NS (On-pump vs Steroid)
Ao clamp time (min)	98 ± 38	108 ± 32	—	NS (On-pump vs Steroid)
No. of bypass	3.9 ± 1.0	3.8 ± 1.2	2.8 ± 1.2	NS
Hemoglobin (g/d*l*)				
Preoperative	11.2 ± 2.3	11.6 ± 2.3	12.1 ± 1.7	NS
Postoperative	10.5 ± 1.0	10.5 ± 1.5	10.7 ± 1.6	NS

NS, not significant; UA, unstable angina; AP, angina pectoris; OMI, old myocardial infarction; AMI, acute myocardial infarction; CPB, cardiopulmonary bypass; Ao, aorta:

evaluated using a Kruskal-Wallis analysis of all groups followed by a Mann-Whitney comparison between individual groups. Correlations between two variables were analyzed by the Pearson correlative coefficient. A one-way repeated-measures analysis of variance (ANOVA) followed by Scheffe's test was used to analyze time-dependent changes. Other data were analyzed by one-way ANOVA followed by Scheffe's test for comparisons among groups. A P value$\leq$0.05 was considered statistically significant.

Results

A total of 163 patients participated in the study. All patients were extubated within 24 h of surgery, and none had significant postoperative complications.

Preliminary Study

One hundred eighteen patients (38 female, 80 male) participated in the preliminary study. Their mean (range) age and weight were 69.4 (50 to 87) years and 59.5 (37 to 87) kg, respectively. Thirty-eight patients underwent CABG without CPB (off-pump bypass: OPCAB), and 41 patients were administered with glucocorticoids during CPB. The mean number of bypasses was 3.5. **Fig. 1** shows the changes in the mean values of CO-Hb concentrations. In all patients and in patients who showed CO-Hb concentrations $< 1\%$ at the preoperative measurement, CO-Hb levels were significantly increased at both the postbypass and the postoperative measurements ($P < 0.05$). However, CO-Hb levels were not significantly changed postoperatively in patients who showed CO-Hb concentrations$\geq 1\%$ at the preoperative measurement.

Main Study

Table 2 shows the demographics and surgical characteristics of the 45 patients. There were no significant differences among groups with respect to gender, age, weight, basal coronary disease, risk factors, anesthesia time, CPB time, and pre- or postoperative hemoglobin concentrations. **Fig. 2** shows the expression ratios of HO-1, HO-2, TNF-α,

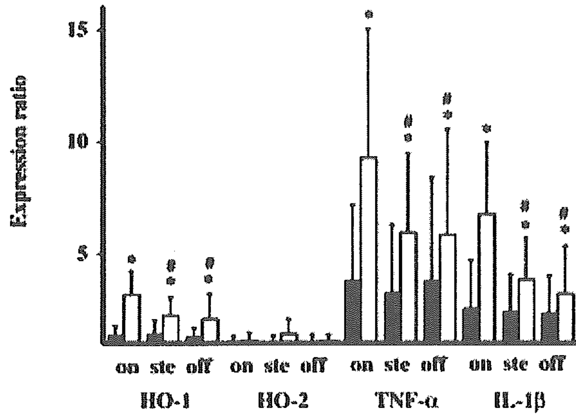

Fig. 2 Expression ratios of hemeoxygenase-1 (HO-1), heme oxygenase-2 (HO-2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) in circulating blood in 45 patients. Black bars = preoperative measurement; white bars = postoperative measurement; on = on-pump group; ste = steroid group; off = off-pump group. *$P<0.05$ vs preoperative measurement; #$P<0.05$ vs the on-pump group.

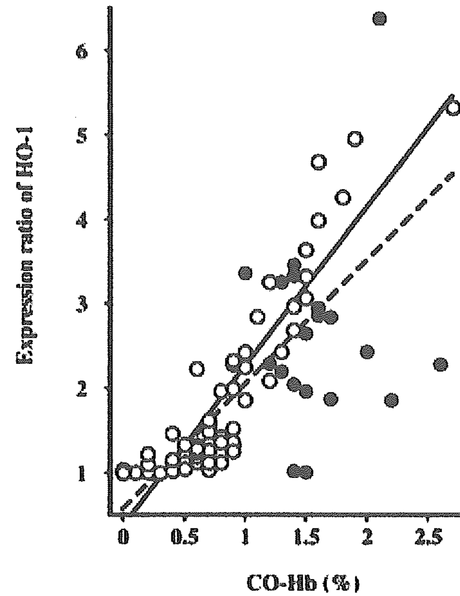

Fig. 3 Relationship between the carboxyhemoglobin concentrations (CO-Hb) and the expression ratio of heme oxygenase-1 (HO-1). Open circles = patients with CO-Hb concentrations < 1.0% at the preoperative measurement (n = 35); closed circles = patients with CO-Hb concentrations ≥ 1.0% at the preoperative measurement (n = 10). Solid line = regression among patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement; dotted line = regression among all patients.

and IL-1β at the preoperative and postoperative measurements in all three groups. The expression ratio of HO-2 did not show any significant changes in any group. The expression ratios of HO-1, TNF-α, and IL-1β significantly increased after surgery in all three groups ($P<0.05$ for all comparisons) and those in the on-pump group were significantly higher than the other groups ($P<0.05$ for all comparisons) at the postoperative measurement.

Fig. 3 shows the relationship between CO-Hb levels and the expression ratio of HO-1 in 90 measurements from 45 patients. There was a significant but weak correlation between these values ($r^2=0.58$); however, a stronger correlation ($r^2=0.80$) was shown when the data from patients whose CO-Hb levels at the pre-operative measurement were ≥ 1% were excluded. No significant correlation between hemoglobin concentrations and CO-Hb concentrations were seen.

There was a significant correlation ($r^2=0.57$) between the expression ratios of TNF-α and HO-1 (**Fig. 4**). There was a significant correlation ($r^2=0.64$) between the expression ratios of IL-1β and HO-1 (**Fig. 5**). The concentration of CO-Hb showed a significant correlation ($r^2=0.69$) with the expression of TNF-α. A weak correlation ($r^2=0.44$) was shown between these values in patients with CO-Hb values ≥1% at the preoperative measurement (**Fig. 6**). The concentration of CO-Hb also showed a significant correlation ($r^2=0.76$) with the expression of IL-1β. A weak correlation ($r^2=0.42$) was shown between these values in patients with CO-Hb levels≥1% at the preoperative measurement (**Fig. 7**).

Discussion

Nitric oxide (NO), which is a gaseous monoxide, was identified as an endothelium-derived relaxing factor in the 1980s, and its various physiologic activities have been proved over the years[19]. Although CO, which is a low molecular gaseous monoxide produced by oxygenase like NO and generated with the heme metabolism in vivo, has

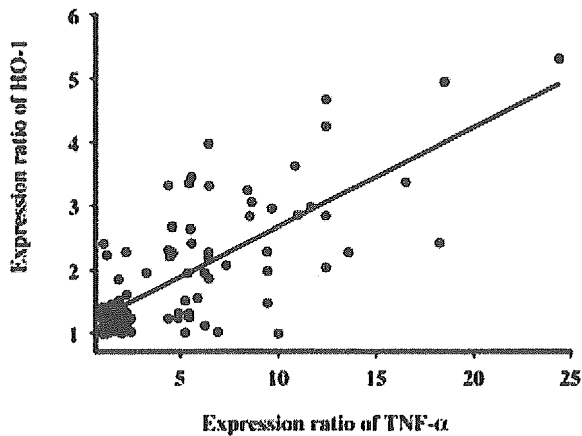

Fig. 4 Relationship between the expression ratios of tumor necrosis factor-α (TNF-α) and hemeoxygenase-1 (HO-1).

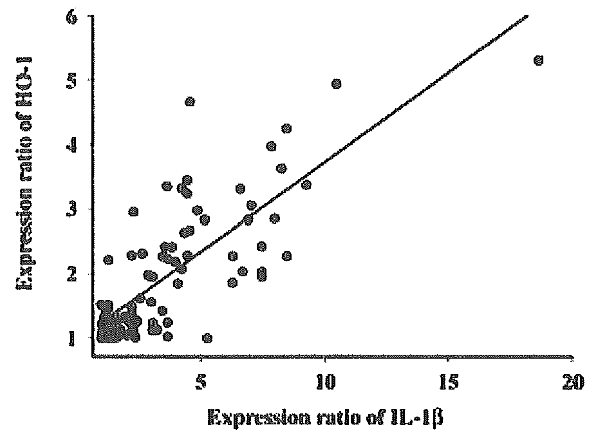

Fig. 5 Relationship between the expression ratios of interleukin-1β (IL-1β) and hemeoxygenase-1 (HO-1).

been known for many years, the physiologic role of CO gained attention in the 1990s after it was reported that CO activates guanylyl cyclase like NO[20]. Endogenous CO is mainly synthesized from HO, and recently, inducible HO-1 has been actively investigated because this enzyme has been shown to have anti-inflammatory, antiapoptotic, antiproliferative, and salutary effects in patients with sepsis[21]. Because CO binds with high affinity to hemoglobin, it is easily measured as CO-Hb by spectrophotometry. Because CO is difficult to metabolize in vivo but is easy to measure, it has become a useful way to evaluate physiologic changes associated with inflammation in vivo.

Locally generated CO is eliminated by hemoglobin in circulating erythrocytes and is gradually released into the alveolar space of the lungs, where molecular oxygen is alternately bound to the heme. Most endogenous-generated CO is thus exhaled into the airway, and the alveolar oxygen tension determines the exchange rate between oxygen and CO[14]. For these reasons, CO-Hb in blood samples collected from patients could be altered by multiple factors such as surgical insults, hemoglobin concentration[10], tissue oxygenation, and pulmonary function[22]. In this study, we chose patients who did not suffer from obvious respiratory or inflammatory diseases, and we fixed the inspired oxygen fraction at 0.5 during the study except for during intubation. We tried to maintain Hb concentrations$>$10 g/dL so that hemoglobin concentrations at sampling time did not differ among groups and were not correlated with CO-Hb concentrations. In spite of the strict patient selection and the fixed patient management, CO-Hb values varied at the preoperative measurement. We also found that changes in CO-Hb concentrations were small in patients who showed CO-Hb concentrations $\geq$ 1% at the preoperative measurement. It is presumed that these patients had functional changes in CO dynamics, such as CO production, binding to Hb, and excretion from pulmonary circulation, or that they already had certain inflammatory changes.

We selected patients undergoing CABG to evaluate changes in CO-Hb concentrations because cardiac surgery with CPB has been recognized to provoke a systematic inflammatory response and the anti-inflammatory strategies, such as administration of corticosteroids[18] or not using CPB[23], were expected to change the inflammation level. Several factors have been assumed to cause the systematic inflammatory response, including contact activation with artificial surfaces of the CPB circuit, reinfusion of shed blood cells, hemodilution, protamine-heparin complexes, operative trauma, and endotoxins released from the temporarily ischemic intestine[24]. Off-pump CABG has been reported to be associated with a lower risk of postoperative morbidity and with significantly lower perioperative serum level of inflammatory cytokines than conventional CABG with CPB[23,24]. Pharmacologic intervention such as administration of