Metabolomic analysis of the mechanism of isoflurane induced apoptosis of PC12 cells

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Abstract

Background: The neurotoxic effects of general anesthetics may adversely affect the developing brain of young animals. Given that levels of reactive oxygen species (ROS) increase in injured mitochondria, the induction of neurotoxicity is likely mediated by oxidative stress. To evaluate this possibility, we conducted metabolomic analyses to identify the metabolites involved in anesthetic-induced neurotoxicity by exposing a well-characterized rat adrenal pheochromocytoma cell line (PC12) to isoflurane.

Methods: PC12 cells were incubated at 37°C in media with serum and then incubated with or without isoflurane for 4 h. After centrifugation, the cells were lysed in 50 μL of water for metabolomics analysis using capillary electrophoresis-mass spectrometry. Metabolite levels were quantitated according to m/z ratios and migration times relative to standards.

Results: Only cells exposed to isoflurane underwent apoptosis. We detected 102 metabolites and identified 91 in isoflurane-exposed cells and control cells. Exposure to isoflurane decreased the levels of reduced glutathione and increased those of oxidized glutathione, indicating that isoflurane accelerated oxidative stress.

Conclusions: These results suggest that oxidative stress mediates isoflurane-induced apoptosis of PC12 cells.

Keywords: Metabolomics, oxidative stress, apoptosis, isoflurane, PC12 cells

Introduction

Evidence accumulated over the past 25 years informs studies of animals that link anesthetics with neurotoxicity. For example, Jevtovic-Todorovic et al., (2003) found that isoflurane administered to rat neonates on postnatal day 7 induces widespread apoptotic neurodegeneration in the developing brain and impaired memory and learning after maturation [1]. Subsequent studies using rodents or monkeys showed that general anesthesia affects the developing brain [2-14]. The question of whether anesthetics induce neurotoxicity in humans has remained controversial, and no conclusive answer has yet been obtained [15,16].

Two studies have implicated mitochondria as mediators of anesthetic-induced neurotoxicity in the developing rat brain [17,18]. Injured mitochondria may generate significant quantities of reactive oxygen species (ROS), suggesting that oxidative stress contributes to the induction of neurotoxicity. The authors of these studies suggested that anesthetics induce oxidative stress by impairing mitochondrial function. However, no comprehensive analysis of anesthetic-induce pathological changes in neural cells has yet appeared.

To address these questions, we conducted a metabolomic analysis to evaluate the effects of an anesthetic on a well-characterized cell line with distinct morphological properties (PC12). We assumed that the identification and quantitation of metabolites produced by PC12 exposed to isoflurane would provide insights into the mechanism of neurotoxicity induced by anesthetics. To our knowledge, this is the first metabolomic analysis of the effect of an anesthetic on a well-characterized animal adrenal pheochromocytoma cell line that serves as a model for neuroendocrine systems.
Materials and methods

Cell culture
PC12 cells, established from a rat adrenal pheochromocytoma, were purchased from the American Type Culture Collection (Catalog # CRL-1721, passage 9; Manassas, VA, USA). They were cultured in growth media: HS (RPMI 1640 containing 10% horse serum and 5% fetal bovine serum (FBS)) and incubated at 37°C in an atmosphere containing 5% CO₂. When 80% confluent, the cells were treated with trypsin for removal from culture dishes and diluted in growth media to 10⁵ cells/mL. Two milliliters of the cell suspension was dispensed into 3-cm dishes. After 16 h incubation, the dishes were washed three times with 2 mL of differentiation media: LS (RPMI 1640 containing 0.1% horse serum and 0.05% FBS). This medium was then replaced with 2 mL of LS containing 100 ng/mL of EGF. After 24 h, 2 mL of isoflurane was added to a first set of 3-cm dishes, which were then placed in a closed container (Tupperware) and incubated for 4 h at 37°C. A second set of 3-cm dishes was placed in a separate closed container and incubated for 4 h at 37°C without isoflurane (control). The cells were observed using a visible-light microscope (Olympus IX73).

Cell viability assay
PC12 cells incubated and collected as described above were diluted with growth medium to 10⁵ cells/mL, and 100-µL aliquots were dispensed into the wells of a 96-well collagen-coated microplate. After 16 h incubation, half of the wells were washed three times with 100 µL of differentiation media, followed by the addition of 100 µL of differentiation medium containing 100 ng/mL of EGF and incubation for a further 24 h. The microplates were placed in a closed container (Tupperware), which was incubated at 37°C. Each row of the microplate was separately sealed with Microseal B Film (Bio-Rad Laboratories, Inc., Hercules, CA). This film seal was then removed in a row-by-row manner at 0, 1, 2, 3, 4, 5, or 6 h, and isoflurane was added at each of these times. After exposure to isoflurane, the cells were incubated for 4 h at 37°C in the presence of 5% CO₂, and the number of viable cells was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Japan).

Apoptosis assay
PC12 cells were seeded in a 3-cm dish, incubated in growth media until the cells reached 80% confluence, exposed to isoflurane for 5 min, and incubated for a further 6 h at 37°C in the presence of 5% CO₂. Cells were collected, and DNA was extracted using a Quick Apoptosis DNA Ladder Detection Kit (BioVision Inc., Milpitas, CA). DNAs were analyzed using 1.2% agarose gel electrophoresis, and the mobilities of the fragments were compared with those of a 100-bp DNA-ladder size marker. Isoflurane-exposed and control cells cultured as above were analyzed for annexin V-binding using an Annexin V-FITC Apoptosis Detection Kit (BioVision Inc.). Annexin V, which is a marker for the early stages of apoptosis, and propidium iodide, which stains dead cells, were detected using a BIOREV fluorescence microscope (Keyence Corp. Japan) and an Accuri C6 flow cytometer (BD).

Metabolomic analysis
PC12 cells were seeded in a 10-cm dish and incubated in growth media until they reached 80% confluence, then exposed to isoflurane (2 ml) for 1 h. We previously confirmed that the results did not change when more than 2 ml was used in a pilot study. The 1 h exposure time was decided from the cell viability assay, and cell viability was maintained for 2 h. The medium was removed, 1.0 mL of methanol was added, and the cells were collected using a cell scraper. Cells were suspended in 1.0 mL of chloroform and 0.4 mL of water, stirred, and centrifuged using an ultrafilter, and the supernatant was removed and dried. The dried supernatant was dissolved in 50 µL of water for analysis.

The quantities and identities of cationic and anionic metabolites present in the samples were determined by capillary electrophoresis-mass spectrometry using an Agilent CE-MS/6224 TOFMS according to the HMT Metabolomics Solution Package (Human Metabolome Technologies, Inc. (HMT), Japan) operating manual. Quantities and identities of 102 metabolites were determined according to their m/z ratios and the migration times of the metabolite standards using the MasterHands metabolomics analysis software (Keio University). The results were mapped to metabolic pathways using the Visualization and Analysis of Networks containing Experimental Data (VANTED) visualization software (http://vanted.ipk-gatersleben.de/). Four independent experiments were conducted.

Statistical analysis
All data are expressed as the mean± standard deviation (SD). Differences in quantities of metabolites between PC12 cells exposed to isoflurane and control cells were evaluated using the Student t test. Statistical differences were not considered, because metabolomics analysis requires multivariate analysis, and the value of determining statistical significance is controversial [19,20].

Results
Effects of isoflurane on cell morphology and viability
Control cells maintained their normal shape and axonal process extensions. In contrast, cells exposed to isoflurane were significantly distorted and disrupted (Figure 1). The axonal processes were indistinct, and many did not exhibit neuronal morphology. After 6 h of incubation, the viability of PC12 cells exposed to isoflurane decreased by 20% compared with control cells, and there was no significant difference in the viability of cells incubated with or without isoflurane that were cultured in differentiation media (LS) or growth media (HS) (Figure 2).

Effect of isoflurane on apoptosis
The DNA of control PC12 cells was largely intact, whereas that of cells exposed to isoflurane was degraded, indicating that
isoflurane induced apoptosis (Figure 3).

Staining with annexin V and propidium iodide, which are indicators of early-stage apoptosis and cell death, respectively, was analyzed using flow cytometry. The absence of staining indicated that the PC12 cells were viable and nonapoptotic (An−PI−). Staining with annexin V alone (An+PI−) indicated that cells were apoptotic, and staining with annexin V and propidium iodide (An+PI+) indicated that cells were dead. The ratios of the number of isoflurane-treated or control cells in each category to the total number of cells are shown in Figure 4. These results indicate that exposure to isoflurane increased the number of apoptotic cells.

**Metabolomic analysis**

Metabolomic analysis of PC12 cells detected 102 metabolites, of which 91 were identified according to m/z values and the migration times of standards (Table 1). These molecules were mapped to the metabolic pathways shown in Figure 5a-5c. In
Table 1. Metabolomic analysis of PC12 cells exposed to isoflurane.

| Metabolites              | Control cells | Isoflurane treated cells | P-value |
|--------------------------|---------------|--------------------------|---------|
| Glyoxylic acid           | ND            | ND                       | --      |
| Glycolic acid            | 0.224         | 0.282                    | 0.3794168 |
| Pyruvic acid             | 3.237         | 2.329                    | 0.5810179 |
| Lactic acid              | 107.751       | 116.900                  | 0.2288466 |
| Fumaric acid             | 1.084         | 1.014                    | 0.7594961 |
| 2-Oxoisovaleric acid     | ND            | ND                       | --      |
| Succinic acid            | 6.243         | 6.964                    | 0.4135116 |
| Malic acid               | 5.250         | 4.764                    | 0.5408992 |
| 2-Oxoglutaric acid       | ND            | ND                       | --      |
| Phosphoenolpyruvic acid  | 0.092         | 0.053                    | 0.91555 |
| Dihydroxyacetone phosphate| 0.045        | 0.025                    | 0.1765466 |
| Glycerol-3-phosphate     | 6.051         | 6.257                    | 0.3484958 |
| cis-Aconitic acid        | 0.053         | 0.049                    | 0.5789018 |
| 3-Phosphoglyceric acid   | 0.168         | 0.086                    | 0.6890028 |
| Citric acid              | 3.836         | 3.624                    | 0.3699757 |
| Isocitric acid           | 0.064         | 0.058                    | 0.4579758 |
| Gluconic acid            | 0.076         | 0.076                    | 0.5980485 |
| Erythrose 4-phosphate    | ND            | ND                       | --      |
| Ribose 5-phosphate       | 0.019         | 0.023                    | 0.3987618 |
| Ribulose 5-phosphate     | 0.716         | 1.004                    | 0.9419956 |
| Fructose 6-phosphate     | 0.095         | 0.045                    | 0.494713 |
| Glucose 6-phosphate      | 0.534         | 0.248                    | 0.4731847 |
| Glucose 1-phosphate      | 0.269         | 0.219                    | 0.0130825 |
| 6-Phosphogluconic acid   | 0.104         | 0.102                    | 0.1731894 |
| Sedoheptulose 7-phosphate| 0.087         | 0.093                    | 0.5983658 |
| dTMP                     | 0.002         | 0.001                    | 0.7671177 |
| CMP                      | 0.254         | 0.166                    | 0.7487105 |
| cAMP                     | 0.110         | 0.094                    | 0.2079311 |
| Fructose 1,6-diphosphate | 1.199         | 0.474                    | 0.990741 |
| cGMP                     | 0.091         | 0.065                    | 0.0174318 |
| AMP                      | 14.335        | 13.083                   | 0.1539805 |
| IMP                      | 2.949         | 2.873                    | 0.4429639 |
| GMP                      | 1.961         | 1.989                    | 0.52431 |
| NADPH                    | 15.131        | 13.833                   | 0.3475598 |
| CoA                      | 7.992         | 9.590                    | 0.0054273 |
| PRPP                     | 0.045         | 0.038                    | 0.2488206 |
| FAD                      | 4.111         | 4.240                    | 0.1058442 |
| dTDP                     | 0.006         | 0.005                    | 0.1212388 |
| CDP                      | 0.046         | 0.029                    | 0.3945933 |
Continuation of Table 1.

| Metabolites                  | Control cells |              |              |              | Isoturane treated cells |              |              | P value |
|------------------------------|---------------|--------------|--------------|--------------|-------------------------|--------------|--------------|---------|
|                              | cell_1        | cell_2       | cell_3       | cell_4       | cell_5                 | cell_6       | cell_7       | cell_8   |
| AcetylCoA                    | ND            | ND           | ND           | ND           | ND                     | ND           | ND           | ND       |
| MalonylCoA                   | ND            | ND           | ND           | ND           | ND                     | ND           | ND           | ND       |
| ADP                          | 3.799         | 3.114        | 5.416        | 3.259        | 2.724                  | 3.219        | 4.310        | 3.473    |
| Succinyl CoA                 | 0.000         | 0.000        | 0.000        | 0.000        | 0.000                  | 0.000        | 0.000        | 0.000    |
| GDP                          | 1.778         | 1.652        | 1.553        | 1.803        | 1.586                  | 1.715        | 1.282        | 1.504    |
| dCTP                         | ND            | ND           | ND           | ND           | ND                     | ND           | ND           | ND       |
| dTTP                         | 0.001         |              |              |              |                        |              |              |          |
| CTP                          | 0.043         | 0.017        | 0.034        | 0.031        | 0.021                  | 0.042        | 0.021        | 0.057    |
| UTP                          | 0.172         | 0.096        | 0.172        | 0.180        | 0.097                  | 0.215        | 0.076        | 0.327    |
| dATP                         | 0.001         | 0.001        | 0.001        | 0.001        | 0.001                  | 0.001        | 0.001        | 0.001    |
| ATP                          | 3.883         | 2.923        | 2.218        | 4.455        | 3.129                  | 4.488        | 1.848        | 4.678    |
| GTP                          | 1.366         | 1.149        | 1.430        | 1.438        | 1.180                  | 1.488        | 0.457        | 1.354    |
| NAD+                         | 3.979         | 3.908        | 3.451        | 4.146        | 3.611                  | 4.070        | 2.821        | 3.259    |
| NADH                         | 40.186        | 46.308       | 40.114       | 38.416       | 40.774                 | 40.570       | 21.907       | 29.728   |
| NADPH                        | 0.103         | 0.108        | 0.113        | 0.116        | 0.085                  | 0.100        | 0.157        | 0.068    |
| Gly                          | 5.778         | 6.515        | 5.508        | 5.161        | 4.825                  | 5.781        | 5.253        | 4.816    |
| Putrescine                   | 0.010         | 0.006        | 0.011        | 0.004        | 0.007                  | 0.005        | 0.012        | 0.004    |
| β-Ala                        | 0.525         | 0.517        | 0.455        | 0.377        | 0.382                  | 0.632        | 0.481        | 0.592    |
| Ala                          | 3.036         | 2.295        | 2.229        | 2.620        | 3.039                  | 2.701        | 2.506        | 2.335    |
| γ-Aminobutyric acid          | 19.003        | 18.521       | 13.309       | 15.188       | 16.768                 | 17.560       | 15.630       | 13.538   |
| Ser                          | 3.359         | 2.542        | 2.268        | 3.357        | 3.245                  | 2.769        | 2.662        | 2.762    |
| Cytosine                     | 0.005         | 0.005        | 0.005        | 0.005        | 0.005                  | 0.006        | 0.004        | 0.003    |
| Uracil                       | 0.072         | 0.095        | 0.076        | 0.073        | 0.069                  | 0.076        | 0.077        | 0.066    |
| Creatinine                   | 0.098         | 0.080        | 0.067        | 0.077        | 0.081                  | 0.068        | 0.066        | 0.056    |
| Pro                          | 0.396         | 0.323        | 0.304        | 0.312        | 0.342                  | 0.344        | 0.314        | 0.363    |
| Val                          | 0.472         | 0.438        | 0.347        | 0.432        | 0.495                  | 0.415        | 0.337        | 0.384    |
| Homoserine                   | 0.036         | 0.024        | 0.028        | 0.026        | 0.027                  | 0.033        | 0.026        | 0.027    |
| Thr                          | 1.731         | 1.560        | 1.513        | 1.584        | 1.530                  | 1.657        | 1.881        | 1.443    |
| Cys                          | 0.026         | 0.030        | 0.012        | 0.019        | 0.017                  | 0.010        | 0.010        | 0.005    |
| Hydroxyproline               | 0.084         | 0.050        | 0.054        | 0.060        | 0.061                  | 0.064        | 0.072        | 0.056    |
| Creatine                     | 32.409        | 25.961       | 23.495       | 24.255       | 28.726                 | 26.938       | 23.091       | 22.525   |
| Ile                          | 0.178         | 0.157        | 0.147        | 0.163        | 0.182                  | 0.165        | 0.124        | 0.125    |
| Leu                          | 0.366         | 0.290        | 0.269        | 0.357        | 0.354                  | 0.326        | 0.260        | 0.288    |
| Asn                          | 0.444         | 0.421        | 0.317        | 0.449        | 0.402                  | 0.353        | 0.360        | 0.232    |
| Ornithine                    | 0.037         | 0.027        | 0.031        | 0.025        | 0.028                  | 0.017        | 0.025        | 0.014    |
| Asp                          | 9.942         | 9.504        | 7.462        | 8.698        | 8.687                  | 8.539        | 7.644        | 7.670    |
| Adenine                      | 0.014         | 0.022        | 0.010        | 0.016        | 0.021                  | 0.015        | 0.013        | 0.007    |
| Hypoxanthine                 | 0.236         | 0.383        | 0.193        | 0.276        | 0.347                  | 0.275        | 0.269        | 0.149    |
| Anthranilic acid             | 0.026         | 0.014        | 0.010        | 0.013        | 0.019                  | 0.015        | 0.010        | 0.009    |
PC12 cells exposed to isoflurane, levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were decreased and increased, respectively. Further, levels of cysteine, which is required for GSH synthesis, were decreased in treated cells compared with control cells. Respective changes in the levels (mean±standard deviation) of cysteine, GSSG, and GSH in PC12 cells before and after exposure to isoflurane were 76.4±19.4%, 147.4±9.5%, and 74.9±12.8% (Figure 6). In contrast, no significant difference in levels was detected between treated vs control cells in levels of the other 88 metabolites. These results show the pathways of apoptosis induced by isoflurane may be simple processes. GSH is an antioxidant produced by cells that protects against oxidative stress. This change in the levels of GSH and GSSG in treated but not control cells was accompanied by an increase in apoptosis. These findings suggest that isoflurane induced direct injuries to mitochondria and do not take other indirect pathways, although further studies are required to confirm this possibility.

**Discussion**

This study is the first metabolomic analysis of the effect of an anesthetic on rat adrenal pheochromocytoma cell line (PC12), which serves as a model for neuroendocrine systems. In this metabolomic analysis, we show that levels of GSH and cysteine (required for GSH synthesis) significantly decreased while those of GSSG increased in PC12 cells treated with isoflurane compared with untreated cells. Moreover, there were no significant differences in levels of the other 88 metabolites. These results show the pathways of apoptosis induced by isoflurane may be simple processes. GSH is an antioxidant produced by cells that protects against oxidative stress. This change in the levels of GSH and GSSG in treated but not control cells was accompanied by an increase in apoptosis. These findings suggest that isoflurane induced direct injuries to mitochondria and do not take other indirect pathways, although further studies are required to confirm this possibility. A limitation of the present study is that the concentration of isoflurane in the cell culture media was not determined. This
Figure 5. Metabolomic analysis of PC12 cells cultured in growth medium (HS).
Metabolites were mapped to metabolic pathways using VANTED software. Exposure to isoflurane decreased cysteine and GSH levels and increased those of GSSG. (a) Glycolysis.
Figure 5. Metabolomic analysis of PC12 cells cultured in growth medium (HS). Metabolites were mapped to metabolic pathways using VANTED software. Exposure to isoflurane decreased cysteine and GSH levels and increased those of GSSG. (b) Pentose phosphate pathway. (c) Nucleic acid metabolism.
was because the isoflurane gas contained in a sealed container was not generated using a vaporizer. Therefore, whether PC12 cells were exposed to clinically relevant concentrations of isoflurane remains to be determined. However, mitochondria in the developing brain are vulnerable to anesthetic agents [17,18,21]. Consistent with this possibility, Jevtovic-Todorovic et al., (2003) showed that inhaled anesthetics significantly decrease mitochondrial density in presynaptic nerve terminals of day-7 postnatal rats [17] and disrupt the balance between mitochondrial fusion and division [18]. Further, they found that Complex IV, which is responsible for the final reaction in the mitochondrial electron transport system, is particularly vulnerable to anesthetics [17]. Abnormalities in Complex IV may allow the leakage of electrons from the mitochondrial electron transport system and may increase ROS production [21]. We therefore consider it reasonable to conclude that oxidative stress induced by inhaled anesthetics injures mitochondria.

Consistent with the hypothesis that isoflurane induces oxidative stress in PC12 cells, Yonamine et al., reported that simultaneous administration of inhaled anesthetics and hydrogen gas decreased neuronal apoptosis in the developing brain and caused subsequent cognitive impairment [22]. Moreover, Boscolo et al., reported that EUK-134, an ROS scavenger, suppressed apoptosis in the brain and prevented the subsequent memory impairment caused by anesthetics [23]. Further, the antioxidant melatonin, which is used to treat insomnia, and the dietary supplement L-carnitine, a selective α2-receptor agonist, as well as the anesthetics dexamethasone and xenon and the mood-stabilizing drug lithium all inhibit anesthetic-induced apoptosis in the developing brain [24-29]. Although the mechanisms underlying these effects are unknown, metabolomics studies such as those presented here may indicate whether they involve oxidative stress.

Conclusions
Our metabolomics analysis shows that the levels of GSH and cysteine decreased significantly while those of GSSG increased in PC12 cells treated with isoflurane compared with untreated cells. The specificity of these effects is remarkable, because there were no significant changes in the levels of 88 other metabolites. These results demonstrate that oxidative stress mediates isoflurane-induced apoptosis of PC12 cells.

List of abbreviations
DNA: Deoxyribonucleic acid
GSH: Reduced glutathione
GSSG: Oxidized glutathione
PC12: Rat adrenal pheochromocytoma cell line
ROS: Reactive oxygen species

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

| Authors’ contributions | KA | KU | MK | ST | HM |
|------------------------|----|----|----|----|----|
| Research concept and design | ✓ | ✓ | ✓ | ✓ | ✓ |
| Collection and/or assembly of data | ✓ | ✓ | ✓ | -- | -- |
| Data analysis and interpretation | ✓ | ✓ | ✓ | -- | -- |
| Writing the article | ✓ | -- | -- | -- | -- |
| Critical revision of the article | ✓ | ✓ | ✓ | -- | -- |
| Final approval of article | ✓ | ✓ | ✓ | ✓ | ✓ |
| Statistical analysis | ✓ | ✓ | ✓ | -- | -- |

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