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-induced 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

Figure 1. Brightfield micrographs of PC12 cells.
(A) Cells in the control group maintained normal neuronal morphology and the extension of axonal processes.
(B) After 4 h of exposure to isoflurane, the morphology of PC12 cells was no longer neuronal.

Figure 2. Viability of PC12 cells cultured in differentiation medium (LS) and growth medium (HS) after exposure to isoflurane.

After incubation for 6 h, the viability of PC12 cells exposed to isoflurane decreased by 20% compared with control cells.

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

100bp
ladder ISO- ISO+

Figure 3. Detection of degraded DNA after exposure of PC12 cells to isoflurane.

Figure 4. Flow cytometric analysis of annexin V- and PI-stained PC12 cells exposed to isoflurane.

An–PI–: viable cells are not stained by annexin V or propidium iodide. An+PI–: apoptotic cells are stained by annexin V alone. An+PI+: dead cells are stained by annexin V and propidium iodide. Comparison of the ratio of the number of cells in each category to the total number of cells showed that the proportion of apoptotic cells increased after exposure to isoflurane.

Table 1. Metabolomic analysis of PC12 cells exposed to isoflurane.

Metabolites	Quantities (nmole/million cells)								P-value
	Control cells				Isoflurane treated cells				
	cell_1	cell_2	cell_3	cell_4	cell_5	cell_6	cell_7	cell_8	
Glyoxylic acid	ND	ND	ND	ND	ND	ND	ND	ND	--
Glycolic acid	0.224	0.282	0.170	0.311	0.368	0.237	0.325	0.231	0.3794168
Pyruvic acid	3.237	2.329	2.528	2.370	1.680	2.078	3.890	1.427	0.5810179
Lactic acid	107.751	116.900	101.820	100.323	108.002	96.523	103.685	83.446	0.2288466
Fumaric acid	1.084	1.014	1.077	0.922	1.089	1.050	1.078	0.772	0.7594961
2-Oxoisovaleric acid	ND	ND	ND	ND	ND	ND	ND	ND	--
Succinic acid	6.243	6.964	5.725	6.288	7.324	5.854	5.194	4.608	0.4135116
Malic acid	5.250	4.764	5.223	4.667	5.174	5.233	4.614	4.074	0.5408992
2-Oxoglutaric acid	ND	ND	ND	ND	ND	ND	ND	ND	--
Phosphoenolpyruvic acid	0.092	0.053	0.083	0.069	0.053	0.082	0.085	0.073	0.91555
Dihydroxyacetone phosphate	0.045	0.025	0.030	0.024	0.032	0.023	0.014	0.013	0.1765466
Glycerol-3-phosphate	6.051	6.257	5.159	5.614	6.501	5.781	3.978	4.085	0.3484958
cis-Aconitic acid	0.053	0.049	0.058	0.049	0.064	0.057	0.045	0.053	0.5789018
3-Phosphoglyceric acid	0.168	0.086	0.149	0.145	0.111	0.122	0.170	0.188	0.6890028
Citric acid	3.836	3.624	4.081	4.089	3.933	4.346	3.881	4.060	0.3699757
Isocitric acid	0.064	0.058	0.060	0.063	0.069	0.065	0.009	0.056	0.4579758
Gluconic acid	0.076	0.076	0.068	0.081	0.085	0.066	0.073	0.064	0.5980485
Erythrose 4-phosphate	ND	ND	ND	ND	ND	ND	ND	ND	--
Ribose 5-phosphate;	0.019	0.023	0.028	0.023	0.028	0.019	0.086	0.018	0.3987618
Ribulose 5-phosphate	0.716	1.004	0.629	0.789	0.929	0.827	0.888	0.532	0.9419956
Fructose 6-phosphate	0.095	0.045	0.062	0.079	0.068	0.045	0.070	0.062	0.494713
Glucose 6-phosphate	0.534	0.248	0.363	0.498	0.421	0.330	0.334	0.348	0.4731847
Glucose 1-phosphate	0.269	0.219	0.168	0.203	0.160	0.133	0.119	0.129	0.0130825
6-Phosphogluconic acid	0.104	0.102	0.085	0.116	0.095	0.091	0.028	0.087	0.1731894
Sedoheptulose 7-phosphate	0.087	0.093	0.075	0.107	0.105	0.081	0.079	0.076	0.5983658
dTMP	0.002	0.001	0.002	0.001	0.002	0.002	0.002	0.001	0.7671177
CMP	0.254	0.166	0.202	0.218	0.209	0.231	0.202	0.225	0.7487105
cAMP	0.110	0.094	0.074	0.084	0.056	0.107	0.066	0.048	0.2079311
Fructose 1,6-diphosphate	1.199	0.474	0.769	1.212	0.932	0.917	1.043	0.752	0.990741
cGMP	0.091	0.065	0.042	0.045	0.016	0.019	0.036	0.009	0.0174318
AMP	14.335	13.083	11.747	12.835	12.602	12.780	11.061	10.501	0.1539805
IMP	2.949	2.873	2.676	3.007	2.932	3.102	2.519	2.304	0.4429639
GMP	1.961	1.989	1.651	2.096	2.094	1.854	1.903	1.340	0.52431
NADPH	15.131	13.833	11.159	14.215	15.240	13.697	0.171	10.947	0.3475598
CoA	7.992	9.590	5.968	7.927	0.915	1.245	5.435	0.198	0.0054273
PRPP	0.045	0.038	0.052	0.037	0.047	0.045	0.052	0.048	0.2488206
FAD	4.111	4.240	3.355	3.924	3.601	3.742	2.375	3.136	0.1058442
dTDP	0.006	0.005	0.005	0.005	0.005	0.005	0.003	0.003	0.1212388
CDP	0.046	0.029	0.045	0.039	0.032	0.043	0.060	0.050	0.3945933

Continuation of Table 1.

Metabolites	Quantities (nmole/million cells)								P value
	Control cells				Isoflurane treated cells				
	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	0.4833225
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	0.1569052
dCTP	ND	ND	ND	ND	ND	ND	ND	ND	--
dTTP	0.001	ND	ND	ND	ND	ND	ND	ND	--
CTP	0.043	0.017	0.034	0.031	0.021	0.042	0.021	0.057	0.7070388
UTP	0.172	0.096	0.172	0.180	0.097	0.215	0.076	0.327	0.7123458
dATP	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.987919
ATP	3.883	2.923	2.218	4.455	3.129	4.488	1.848	4.678	0.8474512
GTP	1.366	1.149	1.430	1.438	1.180	1.488	0.457	1.354	0.3819824
NAD+	3.979	3.908	3.451	4.146	3.611	4.070	2.821	3.259	0.2060684
NADH	40.186	46.308	40.114	38.416	40.774	40.570	21.907	29.728	0.1526851
NADP+	0.103	0.108	0.113	0.116	0.085	0.100	0.157	0.068	0.7136135
Gly	5.778	6.515	5.508	5.161	4.825	5.781	5.253	4.816	0.1702806
Putrescine	0.010	0.006	0.011	0.004	0.007	0.005	0.012	0.004	0.7993839
β-Ala	0.525	0.517	0.455	0.377	0.382	0.632	0.481	0.592	0.4497265
Ala	3.036	2.295	2.229	2.620	3.039	2.701	2.506	2.335	0.6895296
γ-Aminobutyric acid	19.003	18.521	13.309	15.188	16.768	17.560	15.630	13.538	0.7098685
Ser	3.359	2.542	2.268	3.357	3.245	2.769	2.662	2.762	0.9457455
Cytosine	0.005	0.005	0.005	0.005	0.005	0.006	0.004	0.003	0.5331078
Uracil	0.072	0.095	0.076	0.073	0.069	0.076	0.077	0.066	0.3065912
Creatinine	0.098	0.080	0.067	0.077	0.081	0.068	0.066	0.056	0.1907613
Pro	0.396	0.323	0.304	0.312	0.342	0.344	0.314	0.363	0.7809503
Val	0.472	0.438	0.347	0.432	0.495	0.415	0.337	0.384	0.7483729
Homoserine	0.036	0.024	0.028	0.026	0.027	0.033	0.026	0.027	0.932535
Thr	1.731	1.560	1.513	1.584	1.530	1.657	1.881	1.443	0.7822187
Cys	0.026	0.030	0.012	0.019	0.017	0.010	0.010	0.005	0.0480017
Hydroxyproline	0.084	0.050	0.054	0.060	0.061	0.064	0.072	0.056	0.8519385
Creatine	32.409	25.961	23.495	24.255	28.726	26.938	23.091	22.525	0.6482664
Ile	0.178	0.157	0.147	0.163	0.182	0.165	0.124	0.125	0.4707848
Leu	0.366	0.290	0.269	0.357	0.354	0.326	0.260	0.288	0.6888555
Asn	0.444	0.421	0.317	0.449	0.402	0.353	0.360	0.232	0.1882463
Ornithine	0.037	0.027	0.031	0.025	0.028	0.017	0.025	0.014	0.082041
Asp	9.942	9.504	7.462	8.698	8.687	8.539	7.644	7.670	0.2563409
Adenine	0.014	0.022	0.010	0.016	0.021	0.015	0.013	0.007	0.6896277
Hypoxanthine	0.236	0.383	0.193	0.276	0.347	0.275	0.269	0.149	0.8464745
Anthranilic acid	0.026	0.014	0.010	0.013	0.019	0.015	0.010	0.009	0.5168049

Continuation of Table 1.

Metabolites	Quantities (nmole/million cells)								P value
	Control cells				Isoflurane treated cells				
	cell_1	cell_2	cell_3	cell_4	cell_5	cell_6	cell_7	cell_8	
Tyramine	0.002	0.002	0.001	0.001	0.002	0.002	0.002	0.002	0.6241771
Spermidine	0.030	0.025	0.027	0.021	0.025	0.032	0.043	0.017	0.5678377
Gln	18.722	15.457	15.202	15.289	16.270	16.596	14.244	15.405	0.6100686
Lys	0.987	0.845	0.746	0.912	0.936	0.888	0.810	0.815	0.8715895
Glu	23.498	19.739	18.110	19.578	22.146	20.369	19.008	18.247	0.8468057
Met	0.318	0.237	0.287	0.182	0.239	0.268	0.251	0.192	0.6088575
Guanine	0.010	0.014	0.008	0.010	0.010	0.010	0.005	0.008	0.2910125
His	0.479	0.392	0.417	0.373	0.360	0.449	0.339	0.395	0.4081105
Phe	0.461	0.387	0.394	0.299	0.285	0.402	0.354	0.442	0.7664317
Arg	0.563	0.574	0.348	0.486	0.550	0.355	0.323	0.269	0.1917305
Citrulline	0.036	0.027	0.022	0.032	0.039	0.026	0.026	0.023	0.8515974
Tyr	0.563	0.312	0.422	0.374	0.352	0.295	0.390	0.461	0.5185192
DOPA	ND	ND	ND	ND	ND	ND	ND	ND	--
Spermine	0.006	0.012	0.010	0.011	0.012	0.024	0.042	0.011	0.133416
Trp	0.069	0.064	0.053	0.068	0.072	0.070	0.043	0.050	0.5806654
Carnosine	0.553	0.482	0.645	0.410	0.367	0.460	0.428	0.459	0.1360789
Cytidine	0.097	0.082	0.068	0.092	0.097	0.089	0.060	0.061	0.505571
Uridine	0.262	0.355	0.253	0.288	0.285	0.303	0.258	0.244	0.5525246
Adenosine	1.519	1.503	0.873	1.409	1.588	1.240	0.050	0.935	0.343848
Inosine	0.407	0.686	0.451	0.500	0.591	0.510	1.692	0.299	0.4421223
Guanosine	0.090	0.114	0.062	0.093	0.122	0.081	0.076	0.065	0.8229921
Glutathione (GSSG) divalent	1.371	1.332	1.463	1.557	2.078	2.135	1.694	2.351	0.0048195
Glutathione (GSH)	6.483	5.302	3.989	5.089	3.279	3.029	4.291	0.921	0.0367359
S-Adenosylmethionine	0.115	0.100	0.076	0.069	0.092	0.063	0.059	0.047	0.1390027

ND: Not detected. The analysis was repeated four times (n=4). Each analysis detected the presence of 102 unique metabolites, and the identities of 91 were determined using mass spectrometry.

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.

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 6. Changes in metabolite concentrations before and after exposure of PC12 cells to isoflurane.
Changes in the levels of cysteine, GSSG, and GSH in PC12 cells (n=4) treated with isoflurane compared with those of untreated cells (n=4).

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 $\alpha 2$ -receptor agonist, as well as the anesthetics dexmedetomidine 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	✓	✓	✓	✓	✓
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References

- Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW and Wozniak DF. **Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits.** *J Neurosci.* 2003; **23**:876-82. | [Article](#) | [PubMed](#)
- Stratmann G, Sall JW, May LD, Bell JS, Magnusson KR, Rau V, Visrodia KH, Alvi RS, Ku B, Lee MT and Dai R. **Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats.** *Anesthesiology.* 2009; **110**:834-48. | [Article](#) | [PubMed](#)
- Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M and Imaki J. **Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice.** *Anesthesiology.* 2009; **110**:628-37. | [Article](#) | [PubMed](#)
- Fredriksson A, Ponten E, Gordh T and Eriksson P. **Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits.** *Anesthesiology.* 2007; **107**:427-36. | [Article](#) | [PubMed](#)

5. Slikker W, Jr., Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG and Wang C. **Ketamine-induced neuronal cell death in the perinatal rhesus monkey.** *Toxicol Sci.* 2007; **98**:145-58. | [Article](#) | [PubMed](#)
6. Kodama M, Satoh Y, Otsubo Y, Araki Y, Yonamine R, Masui K and Kazama T. **Neonatal desflurane exposure induces more robust neuroapoptosis than do isoflurane and sevoflurane and impairs working memory.** *Anesthesiology.* 2011; **115**:979-91. | [Article](#) | [PubMed](#)
7. Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, Dissen GA, Creeley CE and Olney JW. **Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain.** *Anesthesiology.* 2010; **112**:834-41. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
8. Cattano D, Young C, Straiko MM and Olney JW. **Subanesthetic doses of propofol induce neuroapoptosis in the infant mouse brain.** *Anesth Analg.* 2008; **106**:1712-4. | [Article](#) | [PubMed](#)
9. Johnson SA, Young C and Olney JW. **Isoflurane-induced neuroapoptosis in the developing brain of nonhypoglycemic mice.** *J Neurosurg Anesthesiol.* 2008; **20**:21-8. | [PubMed](#)
10. Palanisamy A, Baxter MG, Keel PK, Xie Z, Crosby G and Culley DJ. **Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults.** *Anesthesiology.* 2011; **114**:521-8. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
11. Bercker S, Bert B, Bittigau P, Felderhoff-Muser U, Buhner C, Ikonomidou C, Weise M, Kaisers UX and Kerner T. **Neurodegeneration in newborn rats following propofol and sevoflurane anesthesia.** *Neurotox Res.* 2009; **16**:140-7. | [Article](#) | [PubMed](#)
12. Liang G, Ward C, Peng J, Zhao Y, Huang B and Wei H. **Isoflurane causes greater neurodegeneration than an equivalent exposure of sevoflurane in the developing brain of neonatal mice.** *Anesthesiology.* 2010; **112**:1325-34. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
13. Callaway JK, Jones NC and Royse CF. **Isoflurane induces cognitive deficits in the Morris water maze task in rats.** *Eur J Anaesthesiol.* 2012; **29**:239-45. | [Article](#) | [PubMed](#)
14. Ibla JC, Hayashi H, Bajic D and Soriano SG. **Prolonged exposure to ketamine increases brain derived neurotrophic factor levels in developing rat brains.** *Curr Drug Saf.* 2009; **4**:11-6. | [Article](#) | [PubMed](#)
15. McCann ME, Bellinger DC, Davidson AJ and Soriano SG. **Clinical research approaches to studying pediatric anesthetic neurotoxicity.** *Neurotoxicology.* 2009; **30**:766-71. | [Article](#) | [PubMed](#)
16. Sanders RD, Hassell J, Davidson AJ, Robertson NJ and Ma D. **Impact of anaesthetics and surgery on neurodevelopment: an update.** *Br J Anaesth.* 2013; **110** Suppl 1:i53-72. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
17. Sanchez V, Feinstein SD, Lunardi N, Joksovic PM, Boscolo A, Todorovic SM and Jevtovic-Todorovic V. **General Anesthesia Causes Long-term Impairment of Mitochondrial Morphogenesis and Synaptic Transmission in Developing Rat Brain.** *Anesthesiology.* 2011; **115**:992-1002. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
18. Boscolo A, Starr JA, Sanchez V, Lunardi N, DiGruccio MR, Ori C, Erisir A, Trimmer P, Bennett J and Jevtovic-Todorovic V. **The abolishment of anesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: the importance of free oxygen radicals and mitochondrial integrity.** *Neurobiol Dis.* 2012; **45**:1031-41. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
19. Noble WS. **How does multiple testing correction work?** *Nat Biotechnol.* 2009; **27**:1135-7. | [Article](#)
20. Dunkler D, Sanchez-Cabo F and Heinze G. **Statistical analysis principles for Omics data.** *Methods Mol Biol.* 2011; **719**:113-31. | [Article](#) | [PubMed](#)
21. Drose S and Brandt U. **The mechanism of mitochondrial superoxide production by the cytochrome bc1 complex.** *J Biol Chem.* 2008; **283**:21649-54. | [Article](#) | [PubMed](#)
22. Yonamine R, Satoh Y, Kodama M, Araki Y and Kazama T. **Coadministration of hydrogen gas as part of the carrier gas mixture suppresses neuronal apoptosis and subsequent behavioral deficits caused by neonatal exposure to sevoflurane in mice.** *Anesthesiology.* 2013; **118**:105-13. | [Article](#) | [PubMed](#)
23. Boscolo A, Milanovic D, Starr JA, Sanchez V, Oklopac A, Moy L, Ori CC, Erisir A and Jevtovic-Todorovic V. **Early exposure to general anesthesia disturbs mitochondrial fission and fusion in the developing rat brain.** *Anesthesiology.* 2013; **118**:1086-97. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
24. Yon JH, Carter LB, Reiter RJ and Jevtovic-Todorovic V. **Melatonin reduces the severity of anesthesia-induced apoptotic neurodegeneration in the developing rat brain.** *Neurobiol Dis.* 2006; **21**:522-30. | [Article](#) | [PubMed](#)
25. Zou X, Sadovova N, Patterson TA, Divine RL, Hotchkiss CE, Ali SF, Hanig JP, Paule MG, Slikker W, Jr. and Wang C. **The effects of L-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex.** *Neuroscience.* 2008; **151**:1053-65. | [Article](#) | [PubMed](#)
26. Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, Fidalgo A, Sun P, Hossain M, Ma D and Maze M. **Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats.** *Anesthesiology.* 2009; **110**:1077-85. | [Article](#) | [PubMed](#)
27. Ma D, Williamson P, Januszewski A, Nogaro MC, Hossain M, Ong LP, Shu Y, Franks NP and Maze M. **Xenon mitigates isoflurane-induced neuronal apoptosis in the developing rodent brain.** *Anesthesiology.* 2007; **106**:746-53. | [Article](#) | [PubMed](#)
28. Straiko MM, Young C, Cattano D, Creeley CE, Wang H, Smith DJ, Johnson SA, Li ES and Olney JW. **Lithium protects against anesthesia-induced developmental neuroapoptosis.** *Anesthesiology.* 2009; **110**:862-8. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
29. Young C, Straiko MM, Johnson SA, Creeley C and Olney JW. **Ethanol causes and lithium prevents neuroapoptosis and suppression of pERK in the infant mouse brain.** *Neurobiol Dis.* 2008; **31**:355-60. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)

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