Laboratory Reports

Hydroxyl radical formation during inhalation anesthesia in the reperfused working rat heart

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Purpose: To determine whether isoflurane, sevoflurane and halothane influenced hydroxyl radical production in the ischemic rat heart.

Methods: Twenty-four male Wistar rats were divided into four groups; control (C), isoflurane 1.4% (I), sevoflurane 2.5% (S) and halothane 1% (H). The hearts were perfused with modified Krebs-Henseleit bicarbonate buffer by a working heart model for 10 min. Then, whole heart ischemia was induced by severely restricting coronary perfusion for 15 min. Reperfusion of the hearts after this ischemic period lasted for 20 min. The coronary effluent was collected before and during ischemia and at 1, 5, 10, 20 min after reperfusion. At the end of reperfusion, hearts were removed and prepared for measurement. Hydroxyl radicals were identified by their reaction with salicylic acid to yield dihydroxybenzoic acids (DHBAs).

Results: Before and after ischemia, there were no differences in coronary flow and heart rate among the four groups, but cardiac output and LV dp/dt maximum in the anesthetic groups were lower than in the control group. Hydroxyl radical products in the heart were significantly lower in the I group than the other groups (e.g. C vs I, 278.1 ± 24.3 vs $219.3 \pm 14.4 \mu\text{M}\cdot\text{g}^{-1}$, $P < 0.05$). The concentrations of DHBAs in the coronary effluent at some points in the I and H groups were less than in the C and S groups.

Conclusion: These results indicate that isoflurane and halothane (to a lesser extent), reduce hydroxyl radical production in the ischemic heart, but sevoflurane does not.

Objectif : Déterminer si l'isoflurane, le sévoflurane et l'halothane ont influencé la production de radical hydroxyle dans des coeurs de rats ischémiques.

Méthode : Vingt-quatre rats mâles Wistar ont été répartis en quatre groupes : témoin (T), isoflurane 1,4 % (I), sévoflurane 2,5 % (S) et halothane 1 % (H). Les coeurs ont été perfusés avec une solution tampon modifiée de bicarbonate Krebs-Henseleit selon un modèle de travail cardiaque pendant 10 min. Ensuite, une ischémie cardiaque totale a été induite en limitant la perfusion coronaire de façon importante pendant 15 min. À la suite de quoi, la reperfusion a duré 20 min. L'effluent coronarien a été recueilli avant et pendant l'ischémie et à 1, 5, 10, 20 min après la reperfusion. À la fin de la reperfusion, les coeurs ont été retirés et préparés pour l'étude. Les radicaux hydroxyles ont été identifiés par leur réaction avec l'acide salicylique pour produire des acides dihydroxybenzoïques (ADHB).

Résultats : Avant et après l'ischémie, il n'y avait pas de différence de flux coronarien et de fréquence cardiaque intergroupe, mais le débit cardiaque et le dp/dt maximal du VG dans les groupes I, S et H étaient plus faibles que dans le groupe T. La production de radical hydroxyle dans le coeur était moins importante dans le groupe I que dans les autres groupes (par ex. C vs I, $278,1 \pm 24,3$ vs $219,3 \pm 14,4 \mu\text{M}\cdot\text{g}^{-1}$, $P < 0,05$). Les concentrations d'ADHB de l'effluent dans les groupes I et H ont été, à quelques reprises, plus faibles que dans les groupes C et S.

Conclusion : L'isoflurane et l'halothane (à un moindre degré) réduisent la production de radical hydroxyle dans le coeur ischémique, contrairement au sévoflurane.

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OXYGEN free radicals are chemical species generated in small amounts during normal cellular metabolism. Cells are normally equipped with endogenous scavenging systems. However, when large amounts of oxygen free radicals are generated during reperfusion of post-ischemic tissues, they can overwhelm cellular defences, and induce tissue damage.¹⁻⁴ Inhalation anesthetics exert cardioprotective effects during myocardial ischemia and reperfusion⁵⁻¹⁰ and this protection is independent of their hemodynamic effects.^{9,10} These results suggest that inhalation anesthetics may decrease free radical formation in the post-ischemic reperfused heart. Yoshida *et al.*¹¹ reported that sevoflurane generated oxygen free radicals, and recent evidence indicates that halothane prevents post-ischemic production of hydroxyl radicals in the canine heart.¹² Thus, it is of interest to investigate the relationship between inhalation anesthetics and oxygen free radical formation in the post-ischemic reperfused heart.

In the present study, we used the ischemic working rat heart preparation to investigate whether inhalation anesthetics affected hydroxyl radical production in the reperfused hearts. We employed a sensitive method to detect hydroxyl radical utilizing high pressure liquid chromatography with electrochemical detection (LCED).^{13,14}

Materials and Methods

The study was approved by the Animal Ethical Committee of the Yamanashi Medical University. Twenty-four three month old male Wistar rats weighing 280-320 g were used. They were randomly divided into four groups as follows: 1. Control (C) group; (n=6) no drugs given during perfusion. 2. Isoflurane (I) group; (n=6) received isoflurane 1.4% during perfusion. 3. Sevoflurane (S) group; (n=6) received sevoflurane 2.5% during perfusion. 4. Halothane (H) group; (n=6) received halothane 1.0% during perfusion. These concentrations of anesthetics were the MAC values for rats.^{15,16} The animals in each group were anesthetized with 5-7 % of each inhalation anesthetic for about seven minutes before their hearts were removed. In the control group, rats were anesthetized with sevoflurane because of its lowest partition coefficient of blood gas solubility. The hearts were then rapidly excised and perfused according to the Langendorff procedure. Non-recirculating modified Krebs-Henseleit bicarbonate buffer was used as preperfusate. The perfusate was maintained at $37.0 \pm 0.3^\circ\text{C}$ and contained (mM): NaCl 118, KCl 4.7, CaCl_2 2.0, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25,

di-NaEDTA 0.5, and glucose 11. The solution was equilibrated with a gas mixture of O_2 95% and CO_2 5%.

During Langendorff perfusion, the left atrium was connected via a pulmonary vein to an angled steel cannula. After this preliminary perfusion, the heart was converted to a working preparation for a stabilization period of 10 min. In this preparation, the perfusate was ejected from the heart to an aortic bubble trap, placed above the heart. The after-load was maintained at a constant level by setting the height of the aortic bubble trap over the heart level (60 mmHg).

Left ventricular pressure was measured with a transducer (P10EZ, Gould, Oxnard, CA, USA) connected to a thin catheter (18G, Argyle Intramedicut Catheter, Sherwood, Tokyo, Japan) inserted into the left ventricle through the mitral valve from the angled steel cannula in the left atrium. Rates of tension development (dP/dt) were measured from the derivatives of left ventricular pressure obtained electronically. Aortic outflow was recorded with an electromagnetic blood flow meter (MFV-3200, Nihon Kohden, Tokyo, Japan). Coronary flow was measured by timed collections of the pulmonary artery outflow and surface run-off of the heart resulting from coronary sinus and Thebesian vessel drainage. Cardiac output was considered as the sum of the aortic and coronary outflows. At no time was the coronary effluent recirculated.

For measurement of oxygen tension of coronary effluent, a catheter was placed in the pulmonary artery. The oxygen tension was measured by an intermittently self-calibrating blood gas analyzer system (Instrumentation Laboratory Model 1306, Lexington, MA, USA). Myocardial oxygen consumption, MVO_2 ($\mu\text{M}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$), was calculated as O_2 solubility multiplied by coronary flow per gram heart tissue multiplied by the difference between inflow O_2 and outflow O_2 tensions. Oxygen delivery (DO_2) was calculated from the inflow O_2 tension multiplied by O_2 solubility multiplied by coronary flow per gram heart tissue.

After initial stabilization (10 min), the heart was exposed for 10 min to the perfusate equilibrated without (control group) or with isoflurane 1.4%, sevoflurane 2.5% or halothane 1.0% in the oxygenating chamber. The concentration of the anesthetic was measured continuously in the gas phase of the oxygenating chamber by an Acoma Anesthetic Agent Monitor (Acoma, Tokyo, Japan).

Then, whole heart ischemia was induced by clamping the one-way aortic valve bypass for 15 min.¹⁷ During the ischemic period, the heart was paced at 333 $\text{beats}\cdot\text{min}^{-1}$. Reperfusion of the heart after ischemia was performed by declamping the one-way aortic valve bypass tube and lasted for 20 min. The

TABLE I Changes in cardiac output and coronary flow.

Time (min)	-10	0	5	10	15	20 (R5)	25 (R10)	30 (R15)	35 (R20)
Cardiac output	ml·min ⁻¹	<	Ischemia		>				
Control	47.7 ± 4.6	48.7 ± 5.1	0.2 ± 0.2†	0.1 ± 0.1†	0.0 ± 0.0†	0.6 ± 1.5†	16.2 ± 12.6†	27.5 ± 6.1†	32.4 ± 5.1†
Isoflurane	46.2 ± 4.5	40.3 ± 5.1*	0.6 ± 1.3†	0.6 ± 1.3†	0.6 ± 1.2†	0.0 ± 0.0†	2.5 ± 6.1*†	10.4 ± 7.6*†	15.8 ± 7.1*†
Sevoflurane	46.4 ± 4.5	39.4 ± 5.4*	0.4 ± 0.3†	0.3 ± 0.3†	0.3 ± 0.3†	0.0 ± 0.0†	4.5 ± 6.7*†	14.6 ± 8.4*†	19.5 ± 8.4*†
Halothane	46.2 ± 1.6	38.8 ± 3.7*	0.4 ± 0.3†	0.3 ± 0.3†	0.3 ± 0.3†	0.0 ± 0.0†	2.7 ± 4.9*†	11.1 ± 5.0*†	17.5 ± 6.6*†
Coronary flow	ml·min ⁻¹								
Control	13.5 ± 0.9	12.8 ± 1.0	0.3 ± 0.1†	0.3 ± 0.1†	0.3 ± 0.1†	7.4 ± 2.1†	9.1 ± 1.7†	10.2 ± 0.8†	10.2 ± 1.3†
Isoflurane	14.5 ± 2.4	14.2 ± 2.4	0.4 ± 0.1†	0.3 ± 0.1†	0.2 ± 0.2†	10.3 ± 3.1†	10.4 ± 2.3†	12.2 ± 2.3	12.2 ± 2.5
Sevoflurane	12.7 ± 1.9	12.8 ± 1.6	0.3 ± 0.1†	0.2 ± 0.1†	0.2 ± 0.1†	10.6 ± 2.9†	10.3 ± 1.6†	11.3 ± 1.4	11.1 ± 1.4
Halothane	13.7 ± 2.0	13.5 ± 2.0	0.3 ± 0.1†	0.3 ± 0.1†	0.2 ± 0.1†	10.1 ± 1.7†	10.7 ± 2.0†	11.9 ± 1.9	12.0 ± 2.0

R5, R10, R15, R20; 5, 10, 15, 20 min after reperfusion, respectively.

Each group; n=6

* $P < 0.05$ vs Control, † $P < 0.05$ vs each value at 0 min.

TABLE II Changes in heart rate, LV dP/dt max and DO₂ to MVO₂ ratio.

Time (min)	-10	0	5	10	15	20 (R5)	25 (R10)	30 (R15)	35 (R20)
Heart rate	beats·min ⁻¹	<	Ischemia		>				
Control	263 ± 57	279 ± 48	333	333	333	43 ± 48†	192 ± 58†	273 ± 55	270 ± 48
Isoflurane	277 ± 27	266 ± 28	333	333	333	3 ± 8†	155 ± 133†	251 ± 56	246 ± 39
Sevoflurane	275 ± 40	274 ± 37	333	333	333	52 ± 62†	152 ± 61†	240 ± 17	238 ± 16
Halothane	279 ± 37	255 ± 26	333	333	333	74 ± 50†	161 ± 61†	242 ± 34	238 ± 28
LV dP/dt max.	mmHg·sec ⁻¹								
Control	4812 ± 651	4653 ± 938	180 ± 64†	70 ± 47†	63 ± 48†	357 ± 398†	3143 ± 583†	3580 ± 348†	3863 ± 475†
Isoflurane	5280 ± 613	4786 ± 675	143 ± 90†	80 ± 100†	80 ± 100†	63 ± 155†	1323 ± 1405*†	3110 ± 426*†	3378 ± 432†
Sevoflurane	4930 ± 857	4243 ± 560	167 ± 63†	130 ± 56†	130 ± 56†	387 ± 449†	2053 ± 739†	2933 ± 344*†	3247 ± 331†
Halothane	4697 ± 1016	4340 ± 525	173 ± 62†	110 ± 60†	80 ± 46†	607 ± 709†	2120 ± 802†	2910 ± 306*†	3287 ± 324†
DO ₂ /MVO ₂									
Control	1.50 ± 0.08	1.45 ± 0.09	0.82 ± 0.64†	0.82 ± 0.63†	0.66 ± 0.72†	1.80 ± 0.25	1.54 ± 0.11	1.52 ± 0.16	1.49 ± 0.08
Isoflurane	1.51 ± 0.07	1.52 ± 0.10	1.28 ± 0.06	1.09 ± 0.54†	0.00 ± 0.00†	1.52 ± 0.14	1.56 ± 0.21	1.53 ± 0.15	1.54 ± 0.09
Sevoflurane	1.54 ± 0.08	1.57 ± 0.05	1.32 ± 0.04†	1.09 ± 0.54†	0.00 ± 0.00†	1.73 ± 0.10	1.63 ± 0.19	1.62 ± 0.09	1.60 ± 0.13
Halothane	1.52 ± 0.08	1.54 ± 0.13	1.26 ± 0.04	1.10 ± 0.54†	0.00 ± 0.00†	2.09 ± 0.33†	1.77 ± 0.37	1.67 ± 0.12	1.63 ± 0.13

DO₂: Oxygen delivery, MVO₂: Myocardial oxygen consumption.

R5, R10, R15, R20; 5, 10, 15, 20 min after reperfusion, respectively.

Each group; n=6 † $P < 0.05$ vs the other groups. * $P < 0.05$ vs Control. † $P < 0.05$ vs each value at 0 min.

administration of inhalation anesthetics continued until the end of reperfusion.

At the end of perfusion, the heart was quickly frozen in liquid nitrogen and was freeze-dried for six days. The tissue was minced into small pieces and homogenised in a polytron. An aliquot was extracted with perchloric acid and centrifuged at 3000 rpm. The supernatant was used for the detection of dihydroxybenzoic acids (DHBAs). A small amount of perfusate was collected from the coronary effluent just before ischemia (0 min), during ischemia (just before reperfusion), 1, 5, 10 and 20 min after reperfusion (i.e. 16, 20, 25 and 35 min after the start of ischemia). The perfusate was also used for the detection of dihydroxybenzoic acids (DHBAs).

Determination of dihydroxybenzoic acids

Hydroxyl radicals react with salicylic acid yielding 2,3-, 2,4-, and 2,5-DHBAs. The liquid chromatography apparatus consists of a Shimadzu Model LC-10AD pump and a detector module (C-R4A, CHROMATOPAC, Shimadzu Co. Ltd. Japan). The column used was a Shim-Pack, CLC-ODS, 15 cm x 4.6 mm. The mobile phase was 90% of 20 mM sodium dihydrogenphosphate, 1 mM octanesulfonic acid sodium salt and 10 mM sodium sulfate, and 10% acetonitrile at a flow rate of 0.5 ml·min⁻¹. The mobile phase was kept anaerobic by N₂ (DEGASSER, DGU-3A, Shimadzu Co. Ltd. Japan). The detector was set at a detector voltage of 0.6 V. All samples were measured against external standards of 2,3-, 2,4- and 2,5-DHBAs and sal-

FIGURE 1 Concentrations of DHBAs in the heart.
* $P < 0.05$ vs the other groups.

FIGURE 2 DHBA levels in the perfusate.
Before; Just before ischemia. Ischemia; Just before reperfusion.
R1, R5, R10 and R20 represent 1, 5, 10 and 20 min after reperfusion, respectively.
† $P < 0.05$ vs the values before ischemia.
* $P < 0.05$ vs the control group.
‡ $P < 0.05$ vs the sevoflurane group.

iclyc acid. Preliminary experiments determined these settings to yield maximal detection ($< 1 \text{ nM} \cdot \text{L}^{-1}$) with minimal interference from other cellular constituents and the variability was within 1%. One millilitre of effluent was treated with 1 ml 1 M Tris buffer and extracted with 2 ml methanol on a Vortex mixer for 10 min. Alumina 50 mg was added to this and the methanol layer was separated. The residue was dissolved in 200 µl of 0.2 M perchloric acid and 5 µl of this solution was injected into the LCED unit. These methods were modified as described by Floyd *et al.*^{13,14}

The data are expressed as mean $\pm$ S.D. Analyses of significant differences in the time-dependent changes

among the four groups were determined by two-way ANOVA with repeated measures, followed by paired *t* tests with the Bonferroni correction. The data of DHBAs among the four groups were analyzed by one-way ANOVA, followed by the Newman Keuls test. A probability of $P < 0.05$ was regarded as statistically significant.

Results

Cardiac output decreased just before ischemia with the three inhalation anesthetics. Moreover, cardiac output in all anesthetic treated groups decreased 10, 15 and 20 min after reperfusion compared with the C group. However, there were no differences in coronary flow and heart rate among the four groups before or after ischemia (Table I). The LV dP/dt maximum in all anesthetic groups was lower than that in group C 15 min after reperfusion.

During ischemia, $\text{DO}_2 : \text{MVO}_2$ ratios in all groups were lower than before ischemia. Five minutes after reperfusion, the $\text{DO}_2 : \text{MVO}_2$ ratio in the H group was greater than in the other groups. However, there were no differences in the $\text{DO}_2 : \text{MVO}_2$ ratio among the four groups during other periods (Table II).

Figure 1 shows the concentrations of total DHBAs in the heart at the end of reperfusion. The concentrations of DHBAs in the I group were lower than those in the other groups. The concentrations of DHBAs in the coronary effluent during ischemia and reperfusion in all groups were higher than those before ischemia at some points ($P < 0.05$). However, DHBA levels in the I and H groups were lower than those in the C and S groups before ischemia and five minutes after reperfusion. In addition, DHBA levels in the I and H groups were lower than in the S group 5, 10 and 20 min after reperfusion. DHBA levels in the S group were higher than those in the C group 20 min after reperfusion (Figure 2).

Discussion

This study demonstrated that isoflurane decreased the DHBA levels in the heart and that isoflurane and halothane reduced those in coronary effluent during pre- and post-ischemic reperfusion. Isoflurane and halothane may reduce hydroxyl radical production. This suggests that the cardioprotective effects of isoflurane and halothane are concerned with free radical involvement. This is consistent with the recent report that halothane prevents post-ischemic production of hydroxyl radicals in the canine heart.¹² However, this is not the case for sevoflurane. Yoshida *et al.*¹¹ reported that sevoflurane selectively impaired endothelium-dependent relaxation in canine mesenteric arteries by

producing oxygen free radicals, mainly due to inactivation of endothelium derived relaxing factor which is now believed to be identical to nitric oxide or related compounds. They suggested the generation of hydroxyl radical from the sevoflurane-delivered bathing media using electron spin resonance spectrometry. This is compatible with our results indicating that sevoflurane increased hydroxyl radical formation in the coronary effluent compared with control 20 min after reperfusion. However, we have recently reported that sevoflurane and isoflurane neither increased nor decreased hydroxyl radical formation in the rat heart-lung preparation.¹⁸ In this case, the type of perfusate (blood vs buffer) seems to have a major influence on the myocardial effects of anesthetics.

Currently, the most popular theory to explain the pathology of oxygen toxicity is that specific damage occurs as a result of the intracellular production of oxygen-derived free radicals in excessive concentrations which overwhelm the natural defenses of the cell. Hydroxyl radicals are highly reactive and one of the most damaging species. The measurement of free radical formation is important in understanding their role in the pathogenesis of post ischemic reperfusion injury. Although the existence of free radicals has been demonstrated with electron spin resonance spectroscopy, this technique requires expensive equipment. Floyd *et al.*^{13,14} have shown that hydroxyl radicals react with salicylic acids producing DHBAs. These reaction products can be detected by LCED. In this study, we modified Floyd's method and could measure the concentrations of 2,3-, 2,4- and 2,5-DHBAs. Our results indicated that DHBAs were detected in the coronary effluent even before ischemia. This implies that the production of hydroxyl radicals always occurred in this preparation under high pressure of oxygen. Moreover, ischemia enhanced their production and anesthetics influenced them.

Cytotoxic oxygen metabolites have been implicated as participants in ischemic cardiac damage, and several studies indicate that reperfusion injury is associated with oxygen free radicals and calcium paradox.¹⁻⁴ While many previous investigations have demonstrated that inhalation anesthetics show cardioprotective effects during myocardial ischemia and reperfusion.⁵⁻¹⁰ In addition, this protection was independent of the hemodynamic effects of the inhalational agents, especially halothane.^{9,10} These may suggest that inhalation anesthetics decrease free radical formation in the post-ischemic reperfused heart. However, the mechanism of this protection remains to be investigated. Coetzee *et al.*⁷ suggested that the beneficial effects of inhalation anesthetics after cardioplegic arrest might be related to the prevention of the reperfusion injury and could not

be ascribed to depression of global myocardial contractile function. Mattheussen *et al.*¹⁹ have assessed the effect of inhalation anesthetics on myocardial metabolism using the ischemic working heart preparation. Although they employed the same experimental model as we did, inhalation anesthetics were administered only during the pre-ischemic period and they did not affect myocardial high energy phosphates at the end of reperfusion. In contrast to their study, our previous study⁸ indicated cardioprotective effects of inhalation anesthetics when they were administered during the reperfusion period. The conflicting results according to the difference of experimental protocol suggest that the presence of inhalation anesthetics in the period of reperfusion may play an important role in the protective mechanism. There are also reports indicating that intracellular calcium control of anesthetics is important for the reduction of calcium paradox.^{7,20}

In this experiment, there are some differences among the inhalation anesthetics. Isoflurane reduced hydroxyl radical production both in the heart and perfusion buffer. Halothane decreased hydroxyl radical only in the perfusate. On the other hand, sevoflurane increased hydroxyl radical production only at 20 min after reperfusion. We do not know the reason, but the hemodynamic effects of isoflurane and sevoflurane were comparable. These findings are consistent with those of *in vivo* studies^{21,22} showing that sevoflurane and isoflurane have comparable hemodynamic effects under aerobic condition. Moreover, we compared the effects of sevoflurane and isoflurane on myocardial contractility and coronary flow during reperfusion, and it was found that the effects of isoflurane and sevoflurane on cardiac function were almost identical. We also calculated the ratio of DO_2 to MVO_2 in the present study to compare the effects of three inhalational agents on O_2 supply-demand relationship. We found that one MAC of inhalation anesthetics had no effect on this ratio compared with the control. Only at five minutes after reperfusion, halothane increased this ratio. It is well known that after a period of ischemia there is a massive hyperemic response in the early phase of reperfusion¹⁷ and it may influence the data on O_2 supply-demand ratio. If there were significant differences in this ratio among the anesthetics, excessive O_2 supply would have affected the concentrations of DHBAs both in the heart and coronary effluent.

In summary, isoflurane and halothane (to a lesser extent), reduce hydroxyl radical production in the ischemic heart, but sevoflurane does not or may increase it. Further investigation is required to clarify the relation between inhalation anesthetics and oxygen free radicals.

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