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Halothane suppresses the increase in intracellular calcium concentration of isolated rat myocytes during hydrogen peroxide perfusion

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Abstract: Ischemia-reperfusion injury is probably caused by the generation of oxygen free radicals. The final common pathway to cell injury may be mediated by intracellular calcium overloading induced by oxygen free radicals. Volatile anesthetics have been shown to improve myocardial function following reperfusion. To determine whether or not oxygen radicals are involved in the mechanism by which volatile anesthetics improve myocardial function following reperfusion, we investigated the effects of hydrogen peroxide (H_2O_2) on the intracellular calcium concentration ($[Ca^{2+}]_i$) in isolated rat ventricular cells. First, the effects of volatile anesthetics, halothane, isoflurane, or sevoflurane, on $[Ca^{2+}]_i$ were studied in the absence of H_2O_2. Next, myocytes were perfused with volatile anesthetics in the presence of H_2O_2. $[Ca^{2+}]_i$ was measured using fura-2, a Ca^{2+}-sensitive fluorescent dye. None of the volatile anesthetics changed $[Ca^{2+}]_i$ in the absence of H_2O_2. In the presence of H_2O_2, $[Ca^{2+}]_i$ gradually increased during H_2O_2 perfusion. Halothane delayed the onset of the increase in $[Ca^{2+}]_i$ induced by H_2O_2, whereas sevoflurane and isoflurane accelerated the onset. Furthermore, sevoflurane caused more pronounced accumulation of intracellular calcium than did halothane and isoflurane. Therefore, the reduction of excessive intracellular calcium accumulation caused by halothane may have beneficial effects on myocardial function following reperfusion.

Key words: Inhalational anesthetics, Myocytes, Calcium ion, Free radicals

Introduction

Oxygen free radicals are highly reactive compounds which cause peroxidation of lipids and proteins, and are thought to play an important role in the pathogenesis of ischemic and reperfusion abnormalities, including irreversible injury, myocardial stunning, and reperfusion arrhythmias [1]. It is also suggested that the increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) induced by oxygen free radicals plays a crucial role in mediating the onset of irreversible cell injury [2–5]. Administration of free radical scavengers during ischemia and reperfusion have been shown to improve myocardial injury [6]. It was also reported that administration of calcium antagonists during ischemia and reperfusion improved the recovery of myocardial function [7]. Volatile anesthetics also have beneficial effects on myocardial function following reperfusion [2,8,9] and these effects of volatile anesthetics may be related to their ability to limit intracellular calcium accumulation. Therefore, it is of interest to investigate the effects of volatile anesthetics on $[Ca^{2+}]_i$ in isolated rat ventricular cells with or without exogenous oxygen free radicals generated by hydrogen peroxide (H_2O_2).

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Materials and methods

Cell isolation

Approval of the Animal Ethical Committee of Yamanashi Medical University was obtained prior to initiating this study. Male Wistar-ST rats (250–300 g) were anesthetized with pentobarbital 50 mg·kg^{-1}, i.p. following the administration of sodium heparin 300 units·kg^{-1}, i.p. The hearts were rapidly excised, attached to a Langendorf apparatus and perfused with a modified Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. The perfusate contained (in mM): NaCl 135, $CaCl_2$ 1.2, KCl 5.4, $MgCl_2$ 1.0, HEPES 10, and glucose 6. The hearts were initially perfused for 3–5 min with a Ca^{2+}-free solution. Then the hearts were perfused with a solution containing collagenase 0.5 mg·ml^{-1} and trypsin 0.3 mg·ml^{-1} for 10–15 min. The ventricles were then removed and chopped, and the

myocytes were dispersed by gentle agitation in oxygenated solution [10]. After isolation, the myocytes were loaded with fura-2 by exposure to 5 μM of the acetoxy methylester of fura-2 (fura-2 AM, Molecular Probes, Eugene, OR, USA) at 37°C for 60 min. Then, fura-2-loaded myocytes were placed in an experimental chamber fitted with a coverslip, which was mounted on the stage of an inverted microscope (IM35, Carl Zeiss, Oberkochen, Germany).

Experimental protocol

First, the effects of volatile anesthetics on $[Ca^{2+}]_i$ were investigated in the absence of H_2O_2. The myocytes were divided into four groups: (1) control group (modified Krebs-Henseleit solution only), (2) halothane group (1.2% halothane) (3) isoflurane group (1.4% isoflurane), and (4) sevoflurane group (3.3% sevoflurane).

Next, the free radical experiments were performed in the presence of H_2O_2. The myocytes were randomly allocated into four groups ($n = 12$, each group): (1) H_2O_2 group (0.1 mM H_2O_2 alone), (2) halothane group (H_2O_2 with 1.2% halothane), (3) isoflurane group (H_2O_2 with 1.4% isoflurane), and (4) sevoflurane group (H_2O_2 with 3.3% sevoflurane). Concentrations of these volatile anesthetics are considered as the MAC values in male rats [11–14]. In a preliminary study, we examined the effect of H_2O_2 on $[Ca^{2+}]_i$ of the isolated myocytes. In the myocytes perfused with the solution containing 0.1 mM of H_2O_2, $[Ca^{2+}]_i$ began to increase at about 10 min after the start of perfusion and reached a plateau at about 15 min. We used 0.1 mM of H_2O_2 for this experiment.

The myocytes in all groups were perfused at a rate of $0.5\,ml \cdot min^{-1}$ with the modified Krebs-Henseleit solution equilibrated with a gas mixture of 95% O_2 and 5% CO_2 for 5 min. At the start of the experiments, perfusion of the myocytes was begun with solution gassed with volatile anesthetics in the presence or absence of H_2O_2. The myocytes were perfused with each solution for 20 min.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured using fura-2. The cells were irradiated with ultraviolet light, and fluorescence of the fura-2-loaded cells was observed using a light microscope. Fura-2 fluorescence at 510 nm emission was obtained with excitation wavelengths of both 340 and 380 nm. Fluorescence images were obtained every 30 s and were digitized at a resolution of 512 × 512 pixels by a computer (ARGUS-100, Hamamatsu Photonics, Hamamatsu, Japan). Fluorescence ratios were then calculated by dividing, pixel by pixel. To determine $[Ca^{2+}]_i$, the in vivo cali-bration method was used according to Li et al. [15]. Then $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz et al. [16];

$$[Ca^{2+}]_i = Kd \times \beta\,(R - R_{min})/(R_{max} - R)$$

where Kd was the dissociation constant for fura-2-Ca^{2+} and taken to be 224 nM [16], β was defined as the ratio of fluorescence at 380 nm with Ca^{2+}-free to Ca^{2+}-saturating conditions, and R was the ratio of fluorescence obtained with excitation at 340 and 380 nm, with the min and max subscripts denoting the ratios obtained under Ca^{2+}-free and Ca^{2+}-saturating conditions, respectively.

Statistical analysis

Results were expressed as means ± SD. Intergroup comparisons were made using one-way analysis of variance following the Duncan's multiple range tests. Intragroup comparisons were made using two-way analysis of variance followed by non-paired *t*-tests with Bonfferoni's correction. $P < 0.05$ was considered statistically significant.

Results

The isolated myocytes were normally elongated (rod-shaped) and quiescent. At the start of the study, $[Ca^{2+}]_i$ in the isolated myocytes was 48.2 ± 7.2 nM. The effects of volatile anesthetics on $[Ca^{2+}]_i$ in the absence of H_2O_2 are shown in Table 1. In the control group, $[Ca^{2+}]_i$ did not change for 20 min. Administration of three volatile anesthetics had no effect on the $[Ca^{2+}]_i$ during the experimental period. In the sevoflurane group, $[Ca^{2+}]_i$ tended to increase, but the effect did not reach significance. There were no significant changes in each group compared to the values at 0 min and there were no significant differences among the groups.

Time courses of $[Ca^{2+}]_i$ perfused with three volatile anesthetics in the presence of H_2O_2 are shown in Fig. 1. The raw data of $[Ca^{2+}]_i$ are shown in Table 2. In the myocytes perfused with the solution containing H_2O_2

Table 1. Effects of halothane, isoflurane, or sevoflurane on $[Ca^{2+}]_i$ in isolated rat myocytes

Time (min)	Control group	Halothane group	Isoflurane group	Sevoflurane group
0	49.1 (28.5)	47.9 (24.5)	48.0 (27.6)	47.7 (24.3)
4	48.8 (27.5)	48.3 (24.3)	48.0 (27.0)	47.2 (25.9)
8	49.1 (28.1)	47.9 (24.7)	47.6 (27.5)	48.8 (28.4)
12	49.1 (28.2)	47.8 (24.8)	48.2 (26.8)	51.5 (26.3)
16	48.6 (27.6)	47.8 (24.3)	46.9 (26.1)	54.1 (29.3)
20	49.7 (27.9)	48.4 (24.9)	47.1 (26.9)	63.0 (30.4)

Data are presented as means with SD in parentheses.

Table 2. Effects of halothane, isoflurane, or sevoflurane on $[Ca^{2+}]_i$ in isolated rat myocytes treated with H_2O_2

Time (min)	H_2O_2 group	Halothane group	Isoflurane group	Sevoflurane group
0	46.3 (25.9)	48.2 (24.5)	46.1 (27.6)	45.1 (24.3)
4	46.3 (25.8)	47.8 (24.3)	46.2 (27.0)	45.2 (25.9)
8	53.1 (26.8)	48.7 (24.7)	81.2 (27.5)*a	79.3 (29.4)*a
12	187.2 (32.8)*	130.7 (38.5)*b	193.4 (34.7)*	182.8 (40.9)*
16	258.3 (41.2)*	256.4 (52.3)*	252.2 (39.8)*	251.8 (42.6)*
20	299.1 (40.3)*	240.9 (62.9)*	291.1 (38.9)*	332.5 (65.0)*c

Data are presented as means with SD in parentheses.
* $P < 0.05$ compared with the values at 0min in each group.
[a] $P < 0.05$ compared with the H_2O_2 and the halothane groups.
[b] $P < 0.05$ compared with the H_2O_2 the isoflurane, and the sevoflurane groups.
[c] $P < 0.05$ compared with the H_2O_2 the halothane, and the isoflurane groups.

alone, $[Ca^{2+}]_i$ began to increase at about 10 min after the start of perfusion and reached a plateau at about 15 min. In the halothane group, $[Ca^{2+}]_i$ increased significantly at 12 min after the start of the perfusion compared with the value at 0 min, whereas $[Ca^{2+}]_i$ in the isoflurane and the sevoflurane groups increased significantly at 8 min. In both the sevoflurane and the isoflurane groups, $[Ca^{2+}]_i$ at 8 min was significantly higher compared with the H_2O_2 and the halothane groups. Furthermore, $[Ca^{2+}]_i$ at 12 min in the halothane group was significantly lower compared with the isoflurane and sevoflurane groups. Twenty minutes after the start of the perfusion, $[Ca^{2+}]_i$ in the sevoflurane group was significantly higher compared with the two other volatile anesthetics.

Discussion

In the present study, halothane, isoflurane, or sevoflurane alone showed no significant changes in $[Ca^{2+}]_i$ of the isolated rat myocytes compared with the control group. This result is in agreement with the result of Wilde et al. [17] who investigated the effect of halothane on resting isolated myocytes. We could not find any reports of the effects of isoflurane or sevoflurane on $[Ca^{2+}]_i$ in the resting state, whereas both isoflurane and sevoflurane suppressed Ca^{2+} influx through a Ca^{2+} pump in previous studies [18–20].

Ischemia-reperfusion injury is generally believed to be caused by the generation of oxygen free radicals, and the final common pathway to cell injury may be mediated by intracellular calcium overloading [1,4,5]. In the presence of H_2O_2, halothane suppressed an increase in $[Ca^{2+}]_i$, whereas sevoflurane and isoflurane did not. This finding may be consistent with the results of Hoka et al. [9] who showed that halothane inhibited the intracellular calcium accumulation during ischemia in guinea pig hearts. Furthermore, sevoflurane caused excessive intracellular calcium accumulation when compared with

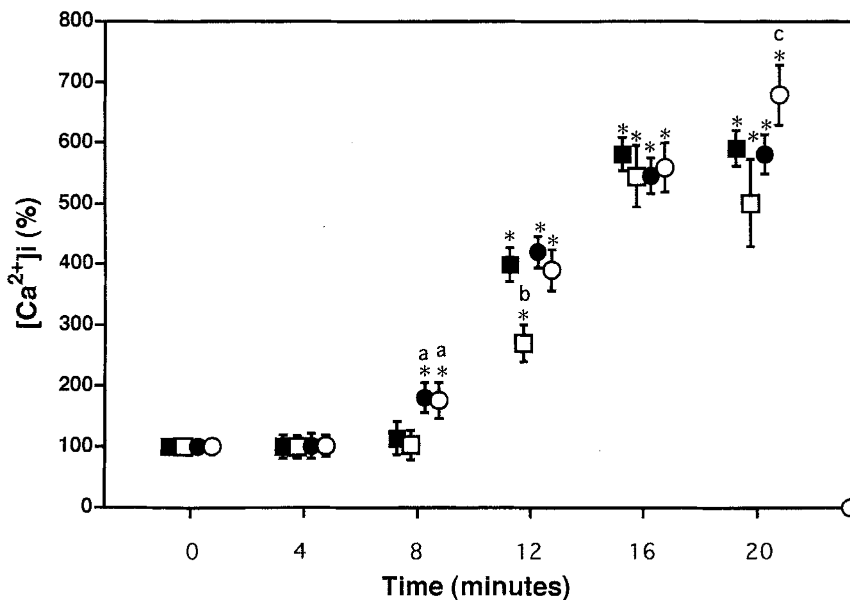

Fig. 1. Effects of halothane, isoflurane, or sevoflurane on $[Ca^{2+}]_i$ in the myocytes in the presence of hydrogen peroxide (H_2O_2). *Closed squares*, H_2O_2 group (H_2O_2 alone); *open squares*, halothane group (H_2O_2 plus halothane); *closed circles*, isoflurane group (H_2O_2 plus isoflurane); *open circles*, sevoflurane group (H_2O_2 plus sevoflurane). Data are given as mean ± SD. *$P < 0.05$ compared with the values at 0 min in each group. [a]$P < 0.05$ compared with the H_2O_2 and the halothane groups. [b]$P < 0.05$ compared with the H_2O_2, the isoflurane, and the sevoflurane groups. [c]$P < 0.05$ compared with the H_2O_2, the halothane, and the isoflurane groups

the other anesthetics. There is a possibility that sevoflurane itself generates oxygen free radicals [21]. This may be related to our result that sevoflurane did not prevent intracellular calcium overloading.

The volatile anesthetics may prevent deleterious intracellular calcium accumulation by attenuating Ca^{2+} influx through slow Ca^{2+} channels. Halothane, isoflurane, and sevoflurane have been demonstrated to inhibit the influx of Ca^{2+} through the slow Ca^{2+} channel [18–20]. Bosnjak et al. showed that this Ca^{2+} channel blocking-like effects were similar in degree for halothane and isoflurane [22]. However, in the current study, isoflurane did not prevent excessive calcium accumulation, whereas halothane did. This may have been due to differences in the methods used in these studies.

Oxygen free radicals also suppressed the sarcoplasmic reticulum Ca^{2+} uptake and led to calcium overloading [23,24]. Stimulation of Ca^{2+} uptake in the sarcoplasmic reticulum may also contribute to a reduction of cytoplasmic Ca^{2+} overloading. The effects of volatile anesthetics on sarcoplasmic reticulum function have remained controversial [17,25]. Therefore, sarcoplasmic reticulum Ca^{2+} uptake may be influenced differently by volatile anesthetics. This may also explain why isoflurane did not attenuate excessive Ca^{2+} overloading.

There are other possible mechanisms for excessive intracellular Ca^{2+} accumulation caused by oxygen free radicals, including inhibition of Na^{+}-K^{+} ATPase in the sarcolemma [26,27], forced Ca^{2+} release from mitochondria [28], and degradation of ion channel proteins of sarcolemmal membranes [29,30]. Whether volatile anesthetics influence these effects caused by oxygen free radicals is uncertain. Further studies are necessary to investigate the effects of volatile anesthetics on free radical injury.

In conclusion, halothane reduced excessive intracellular calcium accumulation induced by H_2O_2 whereas sevoflurane and isoflurane did not.

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