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# EFFECT OF VARIOUS MIXTURES OF DIETHYLETHER, HALOTHANE, NITROUS OXIDE AND OXYGEN ON LOW MOLECULAR WEIGHT IRON CONTENT AND MITOCHONDRIAL FUNCTION OF THE RAT MYOCARDIUM

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Anaesthetic drugs can induce reversible as well as irreversible changes in cell membranes and intracellular proteins as well as lipid peroxidation in the liver. Low molecular weight iron species (LMWI) can by their catalytic activity contribute to the generation of free radicals (hydroxyl radicals). Free radicals are a recognisable cause of intracellular damage. Impaired mitochondrial function is also a sign of intracellular damage, which is usually irreversible. Thus, an agent may be cytotoxic when it causes a significant increase in intracellular LMWI. Whether the LMWI arise from ferritin or is released from iron containing proteins, the same reaction occurs. As long as LMWI can undergo redox cycling, hydroxyl radicals can be formed. We investigated the effect of various mixtures of diethylether, halothane, nitrous oxide and oxygen on the intracellular LMWI content and mitochondrial function of the rat myocardium.

Hearts isolated from rats anaesthetised with diethylether showed an increase in the cytosolic LMWI compared to the control group. No increase in mitochondrial LMWI was demonstrated. Subsequent perfusion of the isolated hearts showed a further increase in the LMWI. On perfusion the mitochondrial LMWI increased in comparison with controls. Mitochondrial function was significantly impaired as measured by the $QO_2$ (state 3), ADP/O ratio and oxidative phosphorylation rate (OPR).

Exposure of rats to 50% nitrous oxide for 15 minutes increased the myocardial LMWI, but had no effect on mitochondrial function. Exposure to room air for 30 minutes before isolating the hearts, still showed a significant increase in LMWI with no detectable change in mitochondrial function.

Halothane, on the other hand, did not have an effect on the myocardial LMWI and mitochondrial function in the experiment setup used. We therefore concluded that diethylether and nitrous oxide are potentially toxic to the myocardium and may potentiate the action of free radicals.

KEY WORDS: Nitrous oxide, halothane, oxygen radicals, mitochondria, iron, diethylether.

## INTRODUCTION

Anaesthetic drugs can induce reversible as well as irreversible changes in cell membranes and intracellular proteins.[1] Evidence is available to support the involvement of free radicals in hepatotoxicity during anaesthesia with halogenated organic compounds. Halothane (1,1,1-trifluoro-2-bromo-2-chloroethane) is widely used as anaesthetic and radicals derived from this drug rapidly react with fatty acids at a rate depending on the degree of saturation.[2] A significant increase in chemiluminescence

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was observed in livers of Wistar rats,[3] as well as increases in diene conjugates (a product of lipid peroxidation)[4] with halothane anaesthesia. Halothane inhibits NADH-CoA reductase, one of the electron transport chain enzymes. This leads to a decrease in ATP production and an increase of NADH. Elevation in NADH is associated with an elevated lactate concentration[5] which can promote low molecular weight iron (LMWI) release from ferritin due to its reducing activity.[6,7]

Intracellular LMWI is potentially toxic as it acts as a catalyst in the Haber–Weiss and Fenton reactions. The end product of these reactions is the highly chemically-reactive hydroxyl radical. Hydroxyl radicals damage important proteins and lipids irreversibly.[8] It is obvious that LMWI must be present for the generation of hydroxyl radicals, and may play an important role in oxygen radical mediated damage of cells. Significant increases in LMWI intracellularly may set the scene for or indicate the presence of free radical induced damage to cellular components.

The inhalation anaesthetic agent diethylether is still used in experimental work.[9,10] Halothane in combination with nitrous oxide ($N_2O$) is popular in modern clinical anaesthesia. Reduced coronary blood flow,[11] ventricular dysfunction[12] and metabolic changes[13] are observed with nitrous oxide in patients with coronary artery disease.

To present no studies have been undertaken to assess the release of LMWI intracellularly by various anaesthetic agents. This study, therefore, was undertaken to determine the effect of diethylether, halothane and nitrous oxide on the LMWI levels and mitochondrial function of the rat myocardium.

## MATERIAL AND METHODS

### *Animals*

Non-pregnant female Wistar rats (300–400 g) were used as experimental animals. Rats had free access to food and water.

### *Experimental design*

*Administration of diethylether.* Rats were anaesthetised in a 2 litre-cage with an inlet and outlet to allow air to circulate. A piece of cotton was wetted with diethylether and put in the cage. As soon as the rats were fully anaesthetised ($\pm$ 5 min) they were decapitated and hearts were removed. One group of hearts served as control, whereas the second group was perfused for 10 min according to the Langendorff technique and 5 min in the working heart mode with Krebs–Henseleit bicarbonate buffer as described before.[14]

*Administration of halothane, nitrous oxide and oxygen.* A conventional anaesthetic machine composing of rotameter units for air, oxygen and nitrous oxide, as well as a Fluotec MK III-halothane vaporizer was used. The breathing apparatus consisted of a 2 litre-glass bottle with a screw-on lid.[15] Fresh gas was supplied through standard anaesthetic tubing mounted onto the lid, at a constant flow rate of 7 litres per minute throughout the experiment. This enabled us to expose rats to various concentrations of oxygen and nitrous oxide with or without addition of halothane.

Three groups of rats were exposed to the following anaesthetic gas mixtures for 15 min: (1) 50% $N_2O$/50% $O_2$ plus halothane, (2) 50% $N_2O$/50% $O_2$ and (3) 50% $O_2$. At the end of the exposure period each group was divided in two subgroups. Rats in

the first subgroup were sacrificed immediately, whereas those in the second subgroup were allowed to breathe air for an additional 30 min. A control group consisted of rats not exposed to halothane, nitrous oxide or 50% oxygen.

After the rats were sacrificed, hearts were removed and homogenised for 4 sec with a Polytron PT (setting 4) in cold buffer (4°C).

*Isolation of mitochondria and mitochondrial function*

Mitochondria were isolated in cold buffer which consisted of 0.18 mM KCl, 0.01 mM EDTA, pH 7.4 with Tris as described previously.[12] Parameters of mitochondrial function determined were the following: (1) $QO_2$ (state 3) (nmol oxygen consumed in the presence of ADP/mg mitochondrial protein/min); (2) ADP/O ratio (nmol ADP consumed/nmol oxygen consumed); and (3) oxidative phosphorylation rate (OPR) (nmol ADP consumed/mg mitochondrial protein/min).[14]

*Determination of LMWI*

A modified cytosolic LMWI method of Krause *et al.*[16] was used. One heart was homogenised in 5 ml cold buffer (4°C) which consisted of 0.25 M sucrose, 5 mM Tris, pH 8.0. After homogenisation the suspension was centrifuged for 60 min at 106 000 g. The supernatant (cytosol) was decanted. A volume of 400 μl 20% TCA was added to 4 ml supernatant, thoroughly mixed and allowed to stand for 10 min, whereafter it was centrifuged for 10 min at 1500 g. To an aliquot of 1 ml supernatant, 0.1 ml 0.1% o-phenanthroline, 0.5 ml 1% ascorbic acid and 0.05 ml saturated ammonium acetate were added. The solution was allowed to stand, for 10 min at 39°C. The absorbance was read at 510 nm against a blank containing all reagents, and the iron concentration calculated by using a standard curve.

*Mitochondrial LMWI*

Mitochondrial pellet (of one heart) isolated as described before was suspended in 2 ml 0.25 M sucrose, 5 mM Tris, pH 8.0. A volume of 200 μl 20% TCA was added and allowed to stand for 10 min, whereafter the same procedure was followed as for cytosolic LMWI.

*Protein*

Protein was determined by the method of Lowry *et al.*[17]

*Statistical analysis*

The values were expressed as the mean ± SEM. Where appropriate the unpaired t-statistics were calculated with the significance level set at $p < 0.05$.

## RESULTS

*Influence of anaesthetic gas mixtures on low molecular weight iron*

Anaesthesia with diethylether caused significant lower mitochondrial oxidative function (Table III) and mitochondrial LMWI as well as a higher cytosolic LMWI content

TABLE I
Effect of diethylether on LMWI content (nmol/mg protein)

| | Rats not subjected to diethylether | N | Rats subjected to diethylether | N |
|---|---|---|---|---|
| *Mitochondria* | | | | |
| Unperfused control | 2.68 ± 0.19 | 6 | 2.08 ± 0.17† | 6 |
| Perfused | 2.74 ± 0.25 | 6 | 3.32 ± 0.19‡ | 6 |
| *Cytosol* | | | | |
| Unperfused control | 0.59 ± 0.02 | 6 | 0.79 ± 0.04†⁰ | 6 |
| Perfused | 0.58 ± 0.04 | 6 | 1.31 ± 0.03‡ | 6 |

Statistical significance: Diethylether vs without diethylether. † < 0.05, ⁰ < 0.001. ‡Perfused vs unperfused control ($p < 0.001$)

TABLE II
Effect of halothane, nitrous oxide and oxygen on cytosolic LMWI iron concentration (nmol/mg protein)

| Group | | 15 min exposure | N | 15 min exposure + 30 min air | N |
|---|---|---|---|---|---|
| (i) | Unperfused control | 0.597 ± 0.02 | 18 | | |
| (ii) | 2% Halothane/ 50% $H_2O$/50% $O_2$ | 0.908 ± 0.031† | 7 | 0.780 ± 0.024† | 6 |
| (iii) | 50% $N_2O$/50% $O_2$ | 1.137 ± 0.08†‡ | 7 | 0.773 ± 0.026† | 6 |
| (iv) | 50% $O_2$ | 0.567 ± 0.02 | 4 | 0.655 ± 0.021 | 4 |

Statistical significance: †vs (i) ($p < 0.001$). ‡(iii) vs (ii) ($p < 0.025$).

TABLE III
Effect of anaesthetics on mitochondrial function

| Group | N | $QO_2$ (state 3) | ADP/O | OPR |
|---|---|---|---|---|
| Unperfused control | 10 | 120.14 ± 4.81 | 2.95 ± 0.18 | 357.28 ± 18.96 |
| Diethylether | 4 | 76.08 ± 11.23† | 3.24 ± 0.07 | 248.20 ± 39.78‡ |
| 2% Halothane/ 50% $N_2O$/50% $O_2$ | 9 | 121.11 ± 3.75 | 2.98 ± 0.02 | 340.81 ± 18.98 |
| 2% Halothane/50% $N_2O$/50% $O_2$ + 30 min air | 8 | 109.21 ± 5.29 | 3.12 ± 0.05 | 361.42 ± 12.77 |
| 50% $N_2O$/50% $O_2$ | 3 | 108.42 ± 7.93 | 3.16 ± 0.08 | 343.63 ± 31.62 |
| 50% $N_2O$/50% $O_2$ + 30 min air | 4 | 108.71 ± 6.48 | 3.16 ± 0.06 | 344.74 ± 22.91 |
| 50% $O_2$ | 5 | 117.78 ± 6.10 | 3.17 ± 0.02 | 365.12 ± 45.05 |
| 50% $O_2$ + 30 min air | 6 | 114.22 ± 5.91 | 3.13 ± 0.07 | 357.33 ± 47.19 |

*Statistical significance: †$p < 0.02$. ‡$p < 0.05$.

in comparison with controls not subjected to diethylether exposure. Perfusion, though, affected LMWI content of rats subjected to diethylether and those not subjected to diethylether differently. The LMWI content of both the mitochondria and cytosol from rats subjected to diethylether increased with perfusion (Table I) while these parameters in the control group stayed unaltered.

With the exception of the 50% oxygen-group where the LMWI concentration was not elevated, the gas mixtures produced a statistically significant increase in LMWI in comparison with the control. A recovery period of 30 min after exposure to the gas mixtures decreased the LMWI. Rats exposed to 50% oxygen prior to the recovery period showed a slight increase in LMWI content (Table II).

*Influence of anaesthetic gas mixtures on mitochondrial function*

Mitochondrial function ($QO_2$ (state 3), and OPR) was significantly damaged in the group subjected to diethylether (Table III), but was not affected by either exposure to halothane, nitrous oxide and/or oxygen or by the thereafter exposure to room air for 30 minutes (Table III).

## DISCUSSION

Iron is stored in ferritin, but must be available for metabolic requirements such as haem synthesis and activation of enzymes which require iron for its catalytic activity. It has been proposed that a small iron pool bound to low molecular weight chelates such as nucleotides, citrate, glycine and glucose exists in the cell. This "transit" pool would enable iron exchange between ferritin, and will provide a source of iron in a form capable of catalysing free radical formation. As many of these LMWI species are known to catalyse free radical reactions *in vitro*, the site of this intracellular pool may be important in determining the level of oxidative stress placed upon the cell.[18]

During myocardial ischaemia cytosolic LMWI increases significantly within 10 min,[19] but the free lysosomal activity (N-acetyl-$\beta$-glucosamidase) stays unaltered.[20] These results suggest that LMWI is not released by proteases. Reperfusion, however, did not elevate LMWI further,[19] but increased free lysosomal enzymes.[20] Desferal, an iron chelator, also inhibits the elevation in free lysosomal enzymes and LMWI may therefore be responsible for the breaking of lysosomal membranes.

Non-haem iron may be released from metalloproteins as indicated by the release of iron from NADH-ubiquinone reductase during myocardial ischaemia.[21] However, whether LMWI is elevated due to an iron release from ferritin or due to proteolytic damage to metalloproteins, an increased LMWI give rise to augmented hydroxyl radical production through its catalytic action in the Fenton reaction.

Our experiments indicated that diethylether anaesthesia depressed myocardial mitochondrial oxidative function in the rat. The impaired mitochondrial oxidative function might be the result of oxygen radicals formed during the Fenton reaction taking place in the cytosol. Because of the elevated LMWI, hydroxyl radicals could be produced in higher than normal concentrations in the cytosol, but not in the unperfused mitochondria. Therefore, these results suggest that mitochondrial damage in this model could come from the cytosolic side. This is not necessarily true for hearts subjected to perfusion, as perfusion elevated both mitochondrial and cytosolic LMWI.

The cause of the elevated LMWI content during diethylether anaesthesia is not known. As diethylether is still used as an anaesthetic agent in animal research work, care should be taken in such experiments.

A combination of various concentrations of halothane, nitrous oxide and oxygen is used regularly. The above-mentioned combination as well as a mixture of nitrous

oxide and oxygen elevated the cytosolic LMWI. Oxygen (50%) without halothane or nitrous oxide did not elevate the cytosolic LMWI. These results suggest that nitrous oxide could be responsible for the elevated LMWI intracellular. Exposure to room air afterwards had a beneficial effect, but could not normalise the LMWI. As the catalyst for Fenton reactions (LMWI) was elevated, hydroxyl radicals might be produced in great amounts. Hydroxyl radicals damage membranes, proteins, DNA, etc. Mitochondrial function, however, was not altered by the above-mentioned gas mixtures although Kashimoto and co-workers[5] found that halothane inhibits NADH-CoA reductase in hearts. The exposure period in our experiments was probably too short to inhibit this enzyme.

Evidence show that nitrous oxide added to other agents was not as innocuous to the circulatory system as previously believed.[11-13,22] When 50% nitrous oxide was added to anaesthesia with either halothane, enflurane or fentanyl in patients undergoing coronary artery bypass grafting, haemodynamic depression occurred. Mean arterial pressure, heart rate, coronary sinus blood flow, as well as systemic vascular resistance decreased. Mean lactate extraction decreased as demonstrated by an increase in coronary sinus lactate content without change in arterial lactate concentration.[11-13] The elevated coronary artery sinus lactate content[11-13] indicated that hypoxia could have occurred causing release of iron from ferritin.[6,7] This suggests a mechanism for the observed elevated LMWI concentration demonstrated in the myocardium as described before. LMWI is potentially toxic, because of its catalytic activity to produce hydroxyl radicals. We support Moffitt and co-workers[11-13] in their opinion that administration of nitrous oxide to patients with coronary artery disease may endanger the myocardium. Although we did not demonstrate mitochondrial damage during or after nitrous oxide administration, it did not mean that other parameters could not have been damaged. Hydroxyl radicals are very reactive and attack different cellular structures.

Koblin and co-workers suggested that nitrous oxide combines with the vitamin $B_{12}$ molecule of methionine synthetase to form a hydroxyl radical that reacts with and inactivates the enzyme. Addition of dimethylthiourea (DMTU), a hydroxyl radical scavenger, prevented the inactivation of methionine synthetase.[22] DMTU would, however, scavenge all hydroxyl radicals, not only those produced by methionine synthetase, but also those formed by LMWI in Fenton chemistry. We, therefore, proposed that cytosolic LMWI might also be important as producer of hydroxyl radicals during nitrous oxide administration.

We conclude that care should be taken where diethylether or nitrous oxide is used as anaesthetic since it elevates LMWI and thereby might produce additional hydroxyl radicals which are able to damage mitochondria.

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