STUDIES ON THE FREE RADICALS IN
MYOCARDIAL MITOCHONDRIA
BY ELECTRON SPIN RESONANCE (ESR) SPECTROMETRY
(STUDIES ON EXPERIMENTAL INFARCTION DOGS)

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FREE radicals are chemical compounds which have one or more unpaired electrons, and can be detected by electron spin resonance (ESR) spectrometry. Biological application of this method was carried out by *Commoner*¹ in 1954. Thereafter, it has been observed that free radicals occurred as intermediates in a number of biological and biochemical systems. *Commoner*²⁻⁴ suggested that free radicals observed in surviving mammalian tissues were due to the free radicals produced as a result of the enzymatic redox activity in mitochondria and that their concentrations were related to the biological origin of the tissue and vary with certain physiological and pathological conditions.

Heart is an organ which is abundant in mitochondria in which aerobic metabolism is actively made and is very sensitive to hypoxic or ischemic conditions. Therefore it is considerable that ESR spectrometry may be useful for the study of myocardial hypoxia.

On the studies of ischemic heart disease, a number of approaches have been used. Experimentally, enzymatic study, polarographic method and electron microscopic study have been used. In the study by coronary sinus catheterization, excess lactate⁵ and Δ redox potential⁶ have been also used as indicators of myocardial

hypoxia. But these indicators are insufficient to reflect the hypoxic state of heart muscle.⁷⁻¹⁰ And ESR spectrometry was introduced as a new approach into the experimental and clinical study of ischemic heart disease by *Yamazaki*⁷ in 1967.

*Akiyama*¹¹ showed that free radicals in heart muscle and coronary venous plasma increased after occlusion of coronary artery of dog. *Yamamoto*¹² observed that free radicals in venous plasma of patients with ischemic heart disease increased after exercise, whereas plasma free radicals of normal subjects decreased after exercise. And the similar findings were observed in experimental coronary insufficiency of dogs produced by Goldberg's method. And clinical application of this method to ischemic heart disease was indicated.

From these data, it is supposed that free radicals may be released from heart muscle into circulating blood in ischemic heart disease, and increased free radicals in heart muscle may be related with the mitochondrial function affected by hypoxia.

In this paper, in order to investigate the free radical formation in myocardial mitochondria and the effect of myocardial ischemia on the free radical formation, following three points were studied.

- 1) Free radical formation in mitochondria of normal dog heart muscle by addition of succinate.
- 2) Effects of aerobic and anaerobic conditions on the free radical formation and destruction.

Key Words:

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3) Effect of myocardial ischemia on the free radical formation of mitochondria by use of experimental infarction dogs.

MATERIALS AND METHODS

Forty adult mongrel dogs were used as experimental animals and divided into two groups. In the first group (12 dogs) no procedure was carried out. In the second group (28 dogs) experimental infarction was produced.

1) Production of experimental myocardial infarction;

Under hexobarbital anesthesia and artificial respiration, thorax was opened through the third intercostal space. After pericardial incision, the anterior descending branch of the left coronary artery was dissected and ligated. And myocardial infarction was confirmed by superficial ECG recording over the area supplied by this vessel. Experimental infarction group was further divided into five groups.

1. 1 hour of coronary artery ligation (10 dogs)

2. 2 hours of coronary artery ligation (6 dogs)

3. 4 hours of coronary artery ligation (4 dogs)

4. After 1 hour of coronary artery ligation, coronary artery was reopened for 5 hours (4 dogs)

5. After 2 hours of coronary artery ligation, coronary artery was reopened for 5 hours (4 dogs)

2) Production of heart particle preparation;

Following procedure was carried out similar to Commoner's method.² Immediately after the resection of pulsating heart, blood was washed out with cold water. The ventricles were removed, cleaned of connective and adipose tissues, and ground with meat-grinder. The resulting macerate was washed by stirring 20 gm of macerate with 200 ml of water for 10 min and the supernatant liquid was poured off gently. This washing procedure was repeated 5 times. Finally the washed macerate was homogenized for 7 min with 50 ml of 0.1 M potassium phosphate buffer at pH 7.4. The resulting homogenate was centrifuged for 30 min at 1800 RCG $\times$ g. The supernatant was retained and the sediment was further homogenized for 3 min with 30 ml of fresh buffer. This second homogenate was centrifuged as above and the first and second supernatants were combined. The combined supernatants were then centrifuged at 16×10^3 RCF $\times$ g for 1 hr. The resulting sedi-

ment was resuspended in a minimal amount of 0.1 M potassium phosphate buffer and used as the heart particle preparation. After grinding the heart, all subsequent procedure was performed at 2–4°C.

3) Production of the preparation for the observation of ESR signal;

10^{-2} M succinate solution (pH 7.4) was added to heart particle preparation and incubated at 37°C, thereafter this suspension was poured into the sample tube made of quartz and frozen in acetone dry ice as quickly as possible. The sample tube was stored at –20°C until ESR observation.

4) Observation of ESR signal;

A JES-ME 3X (Japanese Electron Optics Laboratory Co.) ESR spectrometer was employed in this study. The sample tube was inserted into the Dewar flask filled with liquid nitrogen (–196°C) and measured in the frozen state. Manganese chloride was used as the magnetic field marker and standard substance. Conditions of ESR signal observation are as follows;

1. Resonance cavity: TE 011

2. Microwave: 9410 – 9430 Mc, Power: 0.6 – 0.8 mW

3. Modulation amplitude: 100 Kc, 5.0 Gauss

4. Sweep width: 3300 ± 50 Gauss

5. Scan rate: 100 Gauss/min

6. Response time: 1.0 sec

ESR intensity was estimated from the maximum deflexion of the ESR signals of samples by comparison with the standard signals of manganese chloride inserted in the same resonance cavity of ESR spectrometer.

RESULTS

1. Free radical formation of the heart particle preparation from normal dog heart

Heart particle preparation alone showed no ESR signal at magnetic field in which organic free radicals were observed and succinate solution also showed no signal. However when succinate solution was added to the heart particle preparation and incubated at 37°C, detectable ESR signal was observed at magnetic field of $g=2.003$. (Fig. 1)

ESR intensity from the heart particle preparation isolated from left ventricle, right ventricle and skeletal muscle were 1.40, 1.11 and 0.35 respectively. The signal from left ventricle was the largest in size, while the signal from skeletal muscle was barely detectable. (Fig. 2)

2. Effects of aerobic and anaerobic conditions on the free radical formation and destruction

Fig.1. ESR signals of heart mitochondrial particles (HMP), succinate and mixture of them at liquid nitrogen temperature. Mn represents the signal of manganese chloride as a magnetic field marker. The magnetic field increases to the right along the abscissa.

When the preparation was incubated in the air, the ESR intensity immediately increased during first 3, 5 and 10 min, reached at maximum after 20 min and then gradually decreased. Each ESR intensity was 0.56, 0.64, 0.65 and 0.74 respectively. And after 60 min ESR intensity decreased to 0.22. (Fig. 3)

When the preparation was incubated introducing 20 ml per min of oxygen into this system, the diminution of the signal was much more accelerated and the signal was very nearly undetectable after 60 min.

Under anaerobic condition, on the other hand, which is produced by introducing nitrogen into this system, the signal once produced did not decrease even after 60 min, but remained unchanged on the level of the one after 20 min.

When 10^{-3} M sodium cyanide was added to this system before incubation, decrease of the signal after 60 min was similarly suppressed.

Fig.2. ESR signals of the heart particle preparation isolated from left ventricle, right ventricle and skeletal muscle.

Fig.3. Changes of ESR intensity when the heart particle preparation was incubated in the air at 37°C.

(Fig. 4,5)

When the preparation was boiled for a few seconds, no ESR signals were observed. (Fig. 6)

Fig. 4. Changes of ESR intensity when the heart particle preparation was incubated under the following conditions. 1. introduction of oxygen into the reaction system. (O_2) 2. introduction of nitrogen into the system. (N_2) 3. addition of NaCN to the system. (NaCN)

3. Effects of myocardial ischemia on the free radical formation of heart particle preparation;

In the following studies, ESR intensity from the heart particle preparation isolated from infarcted area was compared with the one from non-infarcted area after succinate solution was added to heart particles and incubated in the air at $37^\circ C$ for 20 min. (Table I, II)

1) 1 hour of coronary artery ligation:

ESR intensity from infarcted area was 0.61 ± 0.15 , while the one from non-infarcted area was 0.73 ± 0.12 . Compared infarcted area with non-infarcted area in the same dogs, ESR intensity from infarcted area was smaller than the one from non-infarcted area in nine cases of 10 cases. ($p < 0.05$) Fig. 7 shows that the ESR signal from infarcted area is smaller than the one from non-infarcted area.

2) 2 hours of coronary artery ligation:

In this group, on the contrary, ESR intensity from infarcted area 0.86 ± 0.10 was larger than the one from non-infarcted area 0.69 ± 0.05 . In the same dogs ESR intensity from infarcted area was larger than the one from non-infarcted area in all cases. ($p < 0.02$)

3) 4 hours of coronary artery ligation:

ESR intensity from infarcted area (0.57 ± 0.16) was again smaller than the one from non-

Fig. 5. ESR signals of the heart particle preparation under the following conditions. 1. in the air (Air) 2. in oxygen (O_2) 3. in nitrogen (N_2) 4. addition of NaCN (NaCN)

infarcted area (0.73 ± 0.15). ($p < 0.1$)

4) When coronary artery was reopened and coronary flow was maintained for five hours after one and two hours of coronary ligation, no difference could be seen between the ESR intensity from infarcted area and from non-infarcted area.

DISCUSSION

According to Commoner² general properties of heart particles are as follows. Spectrophotometrically heart particles contain the $605 m\mu$ α band of cytochromes of the a group, the $560 m\mu$ peak representative of the fused α band of other cytochromes, and the strongly overlapping and mutually repressed peaks at lower wavelengths due to flavins and the γ band of the cytochromes. The heart particles exhibit succinoxidase and succinate-cytochrome c reductase activities, and therefore those components of the electron transport system which effect the transfer of electrons from succinate to cytochrome c and to

TABLE I EFFECT OF CORONARY OCCLUSION ON THE ESR INTENSITY OF HEART PARTICLE PREPARATION

1 hour of coronary artery ligation				2 hours of coronary artery ligation				4 hours of coronary artery ligation			
Case No.	Non-inf	Inf	(Non-inf) -(Inf)	Case No.	Non-inf	Inf	(Non-inf) -(Inf)	Case No.	Non-inf	Inf	(Non-inf) -(Inf)
1	0.82	0.43	0.39	11	0.63	0.73	-0.10	17	0.57	0.34	0.23
2	0.72	0.62	0.10	12	0.70	0.82	-0.12	18	0.68	0.50	0.18
3	0.96	0.82	0.14	13	0.65	0.90	-0.25	19	0.68	0.67	0.01
4	0.59	0.50	0.09	14	0.67	1.04	-0.37	20	0.98	0.76	0.22
5	0.64	0.35	0.29	15	0.80	0.87	-0.07				
6	0.55	0.53	0.02	16	0.67	0.81	-0.14				
7	0.76	0.70	0.06								
8	0.70	0.78	-0.08								
9	0.84	0.78	0.06								
10	0.68	0.62	0.06								
$\bar{x}$	0.73	0.61	0.12		0.69	0.86	-0.17		0.73	0.57	0.16
SD	0.12	0.15			0.05	0.10			0.15	0.16	
SE	0.04	0.05			0.03	0.04			0.09	0.09	
			($p < 0.05$)				($p < 0.02$)				($p < 0.1$)

Non-inf: non-infarcted area
Inf: infarcted area

oxygen are functional.

In this study, neither heart particle preparation nor succinate solution alone produced any free radicals independently. However, when succinate was added to heart particle preparation and incubated at 37°C, clearly detectable ESR signal could be observed at magnetic field of $g = 2.003$. These data suggest that there is close relation between mitochondria and free radical formation.

Under aerobic conditions, free radicals once produced gradually decreased with time, and this phenomenon was more remarkable after sufficient oxygenation. These phenomena, as in Commoner's report,² can be recognized as the result of exhaustion of succinate in the preparation. Under anaerobic condition, on the other hand, the free radicals maintained the concentration even after 60 min, because succinate is not completely oxidised and the system exists in

Fig.6. Effect of heating on the ESR signals of the heart particle preparation (100°C, for a few seconds).

TABLE II EFFECT OF REOPENING OF CORONARY OCCLUSION ON THE ESR INTENSITY OF HEART PARTICLE PREPARATION

Reopening for 5 hours after 1 hour of coronary artery ligation				Reopening for 5 hours after 2 hours of coronary artery ligation			
Case No.	Non-inf	Inf	(Non-inf) -(Inf)	Case No.	Non-inf	Inf	(Non-inf) -(Inf)
21	0.71	0.85	-0.14	25	0.80	0.59	0.21
22	0.74	0.77	-0.03	26	0.66	0.67	-0.01
23	0.84	1.00	-0.16	27	0.77	0.92	-0.15
24	1.11	1.00	0.11	28	0.82	0.76	0.06
$\bar{x}$	0.85	0.91	-0.06		0.76	0.74	0.02
SD	0.16	0.10			0.06	0.12	
SE	0.09	0.06	(n.s.)		0.04	0.07	(n.s.)

Fig.7. ESR signals of the heart particle preparation isolated from infarcted and non-infarcted area. (60 min after coronary occlusion).

equilibrium. In case of addition of sodium cyanide as an electron transfer inhibitor to this system, the similar finding was observed.

Commoner⁴ reported that all free radicals

observed in animal tissues with the exception of those containing melanin, which is a stable free radical, disappeared irreversibly when the tissue was boiled for 5 min. In this study, ESR signals

in the heart particles similarly disappeared when the preparation was boiled for 5 min. Such decay of the free radicals by heating may be due to the destruction of enzymatic activity of heart particles.

These data suggest that free radicals arise and disappear as intermediates in succinoxidase system of mitochondria and the concentration is determined by various kinds of conditions which influence this system. And it is considerable that under the conditions which inhibit electron transport system, the destruction process of free radicals is disturbed.

Free radical formation is far more active in the heart particles than in the mitochondria of skeletal muscle. And according to *Commoner*,³ there is close correlation between the concentration of free radicals and heart particles. There are also the report^{2,13} that the free radical content which was obtained from tissues of several animals declined in order liver > kidney > heart > skeletal muscle. From these data it is supposed that free radical formation may reflect the function of oxidoreductive enzymes in mitochondria and therefore the observation of free radicals may be useful for the evaluation of mitochondrial function.

From this point of view, in order to study the mitochondrial function by use of ESR spectrometry, ESR intensity was compared in infarcted area with non-infarcted area after addition of succinate and incubation at 37°C for 20 min.

*Sands and Beinert*¹⁴ reported that four principal types of signals were observed in the process of oxidation-reduction of purified mitochondrial electron transport enzymes;

1. Symmetrical signals of $g = 2.00$ which were seen on partial reduction of all preparations which contained flavoprotein component. These signals were attributable to flavin semiquinones.

2. A signal at $g = 2.05$ was observed principally in cytochrome oxidase preparations.

3. A signal at $g = 4.3$ which was thought to be due to non-heme iron.

4. An asymmetric signal at $g^{\parallel} = 2.00$, $g^{\perp} = 1.94$ which appeared on reduction of a variety of DPNH or succinic dehydrogenase preparations by their respective substrates.

*Hollocher and Commoner*³ reported as follows. The free radicals observed in their study arise from redox equilibria involving succinate and one or more components of the succinoxidase system which function on the reducing side of the site of

Antimycin A inhibition. The possible complexes are succinate, fumarate, succinic dehydrogenase and cytochrome b. From their experimental observations the free radicals arise only as redox intermediates in the equilibria among succinate, fumarate and succinic dehydrogenase, all of which are regarded as 2-electron donor-acceptors. As an intermediate, a free radical "Fumarate-Flavin-Fe⁺⁺⁺-Fe⁺⁺" is conceivable, because iron atoms which are contained in succinic dehydrogenase may mediate electron exchange among the complexes. And the other possibility that the free radicals arise from an interaction between succinic dehydrogenase and a 1-electron donor-acceptor, such as cytochrome, was ruled out.

The configuration of the ESR signal observed in this study is slightly different from the one of flavin semiquinones. And therefore free radicals observed in this study may be composed of flavin semiquinone, substrate free radical and "Fumarate-Flavin-Fe⁺⁺⁺-Fe⁺⁺".

From the study of respiratory control by *Morita*,¹⁵ disturbance of mitochondrial respiration was already observed 15 min after coronary occlusion. This phenomenon was more remarkable after 60 min. In the mitochondria isolated from the infarcted area of the heart at 60 min after coronary occlusion, respiratory control ratio decreased as a result of increased oxygen consumption in state 4 respiration. On the other hand, oxygen consumption in state 3 did not change. This indicates uncoupled oxidative phosphorylation.

About oxygen consumption and ESR study of mitochondria, there is a report by *Odashima*.¹⁶ When sodium succinate as substrate was added to state 2 respiration, the concentration of free radicals increased about 1.5 fold of state 2. On addition of ADP in state 3 respiration, the concentration remarkably decreased constantly with reaction time. At the end of state 3, the concentration decreased less than a half of that at the initiation of state 3. In state 4 respiration after exhaustion of ADP, an increase in the concentration was observed. And free radicals in state 4 respiration were decreased remarkably by dinitrophenol as an uncoupler.

According to histochemical study by *Mikami*,¹⁷ phosphorylase and cytochrome oxidase activity begin to decrease after 90 min of coronary occlusion, but succinic dehydrogenase activity does not decrease until 180 min after.

According to the electron microscopic study by *Kawamura*,¹⁸ already after 5 min of coronary

occlusion, partial destruction of cristae and disappearance of matrix can be observed. After 30 min disappearance of cristae and matrix rarefaction is remarkable. But ultrastructure of mitochondria is reversible if coronary ligation is released and coronary flow is maintained even after 60 min of coronary occlusion.

Making these observations into consideration, the data in this study may be interpreted as follows. Lowered ESR intensity from the heart particles isolated from infarcted area after 60 min of coronary occlusion agree with the studies of *Morita*, *Odashima* and *Mikami*. after 60 min of coronary occlusion, oxydative phosphorylation is uncoupled but each enzyme activity of electron transport system is still not affected. Therefore the velocity of enzyme catalyzed reaction in respiratory chain is accerelated and the destruction of free radicals once produced is increased. As the result free radical concentration in heart particles decreases. After 120 min of coronary occlusion, on the other hand, because enzyme activity such as phosphorylase and cytochrome oxidase is lowered, the reaction velocity in respiratory chain slows down and the destruction of free radicals is suppressed. As the result free radical concentration in heart particles increases. After 4 hours of coronary occlusion, because enzymatic activity in respiratory chain is remarkably lowered, free radical formation itself is disturbed and free radical concentration in heart particles again decreases. When coronary artery was reopened 60 and 120 min after coronary ligation, no difference of free radical concentration was observed between infarcted and non-infarcted area. These data suggest that mitochondrial function is reversible even after 120 min of coronary occlusion, if coronary ligation is released and coronary flow is maintained.

In ischemic heart disease, free radicals may increase in the mitochondria affected by hypoxia. As shown in the studies by *Akiyama*, *Yamamoto* and *Naito*, free radicals in heart muscle and venous plasma increased in ischemic heart disease. It is obscure whether the free radicals run out into circulating blood or not. But there may be certain relation between free radicals produced in the mitochondria and the free radical concentration in circulating blood. Therefore it is conceivable that observation of the free radicals in plasma is useful for the supposition of the mitochondrial oxidoreductive function and the diagnosis of ischemic heart

disease.

At present preparing method of biological materials is complicated and quantification of the free radicals in biological materials is not necessarily established. But when these procedures are established, clinical application of ESR spectrometry will become to be available.

CONCLUSION

In order to investigate the pathochemical aspects of ischemic heart disease, free radical formation in myocardial mitochondria and the effect of myocardial ischemia were studied by use of ESR spectrometry.

1. No ESR signal was observed in heart particle preparation itself.
2. When succinate was added to heart particles and incubated at 37°C, clearly detectable ESR signal ($g=2.003$) was observed.
3. Under aerobic conditions free radicals once produced gradually decreased, while under anaerobic conditions free radicals maintained the concentration.
4. One hour after coronary occlusion ESR intensity produced by heart particles was lower in infarcted area than non-infarcted area.
5. Two hours after coronary occlusion ESR intensity was higher in infarcted area than in non-infarcted area.
6. Four hours after coronary occlusion ESR intensity was again lower in infarcted area than in non-infarcted area.
7. When coronary artery was reopened and coronary flow was maintained for 5 hours after 1 and 2 hours of coronary ligation, no difference was observed in ESR intensity between infarcted and non-infarcted area.

Free radicals observed in this study arise as intermediate metabolites in electron transport system in mitochondria. And the concentration is determined by the integrity of production and destruction which is regulated by the reaction velocity in respiratory chain. Therefore the concentration of free radicals is decreased by the conditions which induce the uncoupling of oxidative phosphorylation and the conditions which inhibit the respiratory chain, while the concentration is increased by the conditions which moderately suppress the respiratory chain.

These results suggest the ESR spectrometry may be useful for the investigation of myocardial metabolism of ischemic heart disease.

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