Effect of Nitroglycerin on the Free Radical Formation of Myocardial Mitochondria Impaired by Ethanol

Studies by Electron Spin Resonance (ESR) Spectrometry

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SUMMARY

In order to investigate the direct metabolic effect of nitroglycerin on myocardium, its effect on mitochondrial free radical formation was studied in vitro, using the dog heart mitochondria impaired by ethanol. Free radical concentrations in state 4 respiration or the ratio of free radical concentrations in state 4 to state 1 was used as an index of mitochondrial function. Free radical concentrations were represented as ESR intensity.

In normal control, ESR intensity increased from 0.115 ± 0.030 (mean $\pm$SD) per mg protein in state 1 to 0.178 ± 0.017 in state 4 ($p<0.001$). When mitochondria were treated with 10^{-4}M ethanol, on the other hand, ESR intensity decreased from 0.163 ± 0.34 in state 1 to 0.137 ± 0.019 in state 4 ($p<0.05$). When mitochondria were treated with 10^{-6} M nitroglycerin, in spite of the presence of 10^{-4} M ethanol, ESR intensity increased from 0.124 ± 0.23 in state 1 to 0.153 ± 0.024 in state 4 ($p<0.05$). ESR intensity in state 1 was increased by the treatment of ethanol ($p<0.05$), whereas ESR intensity in state 4 was decreased ($p<0.001$), and these effects were prevented by nitroglycerin ($p<0.05$). The ratio of ESR intensity in state 4 to state 1 demonstrated more remarkably the effects of ethanol and nitroglycerin. Ethanol reduced the ratio from 1.65 ± 0.47 in the control to 0.86 ± 0.13 ($p<0.001$) and the reduction was prevented by nitroglycerin to 1.25 ± 0.23 ($p<0.01$).

It is concluded that nitroglycerin prevented the impairment of free radical formation of the mitochondria induced by ethanol, presumably by a mitochondrial membrane-stabilizing action.

Additional Indexing Words:

Respiratory control ratio Free radical concentrations in state 4 respiration The ratio of free radical concentrations in state 4 to state 1

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ALTHOUGH the utility of nitroglycerin in angina pectoris is well established, the mechanism of its action is not fully understood. According to the present view, its action is attributed partly to the reduction in preload and afterload that results in a diminution in myocardial oxygen demands,[1)-6)] and partly to the redistribution of regional myocardial blood flow in ischemic areas.[7),8)] In spite of extensive researches of hemodynamic effects of the drug,[1)-8)] little attention has been focuced on the direct metabolic effects.[9)-11)]

Free radicals are chemical compounds that have one or more unpaired electrons, and can be detected by electron spin resonance (ESR) spectrometry. Free radicals occur as intermediates in electron transport system, when succinate is added to mitochondrial preparation.[12)-14)] Free radical concentrations in state 4 respiration or the ratio of that in state 4 to state 1 have been reported to reflect the efficiency of oxidative phosphorylation in the mitochondria and suggested to be a more sensitive index of the mitochondrial function than respiratory control ratio.[15)-18)]

The purpose of this study was to investigate the direct metabolic effect of nitroglycerin on myocardial mitochondria, using a new method of measuring free radical formation of the mitochondria.

Materials and Methods

Eight mongrel dogs weighing 8 to 12 Kg were anesthetized with intravenous sodium pentobarbital (30 mg/Kg). Ventilation was maintained by means of a Harvard animal respirator with room air. A left thoractomy was performed through the 4th intercostal space, pericardium was opened and the heart was removed from the animals.

Isolation of mitochondria

Immediately after the resection of pulsating hearts, blood was washed with cold water. Left ventricles were removed, cleaned off connective and adipose tissues and minced with meat mincer. Myocardial mitochondria were isolated by the method of Chance and Hagihara.[19)] The minced tissue 10 Gm was treated with alkaline protease (2.5 mg/g of heart) in 100 ml of mannitol solution for 20 min at 4°C. Mannitol solution contained 0.21 M mannitol, 0.07 M sucrose and 0.01 M EDTA, and was ajusted to pH 7.4 with Tris base. The macerate was gently homogenized with teflon homogenizer and the homogenate was allowed to stand for 20 min at 4°C. Then the homogenate was diluted with additional 100 ml of mannitol solution and homogenized again. The resulting homogenate was centrifuged at 500 g for 5 min at 4°C. The supernatant was centrifuged at 12000 g for 10 min at 4°C. Taking off white fluffy layer on the top of the sediment, lower brown layer was resuspended to 30 ml of mannitol solution and centrifuged at 8000 g for 5 min at 4°C. This procedure was repeated twice and the resulting sediment was used as mitochondrial samples. Mitochondrial protein was determined by biuret method.

Determination of mitochondrial respiration

Mitochondrial rspiration was measured polarographically on a oxygen analyzer supplied with Beckman oxygen electrode. Basic reaction medium contained 0.3 M mannitol, 0.01 M KCl, 0.01 M KH_2PO_4, 2.5 mM $MgCl_2$, and 0.25 mM EDTA, and was ajusted to pH 7.4 with Tris base. Succinate, in a concentration of 0.2 M (pH 7.4), was used as a substrate. The reaction was started by addition of 0.1 ml mitochondrial suspension (10–20 mg protein) to 9.4 ml of reaction medium (state 1). Then 0.5 ml of 0.2 M succinate was added (state 4) and 20 μl of 0.1 M ADP was added (state 3). The reaction temperature was 25°C. The respiratory control ratio was defined as the ratio of the respiratory rates in the presence (state 3) and in the absence of ADP (state 4).[20]

The respiratory control ratio of the mitochondria isolated from normal heart was 6.75±0.50 (mean±SD). When the mitochondria were treated with ethanol for 1 min prior to the addition of succinate, ethanol had no effect on the respiratory control ratio at 10^{-4} M and lower concentrations (Fig. 1). From the result of this preliminary study, the effect of nitroglycerin on free radical formation of the mitochondria was evaluated at a concentration of 10^{-6} M, containing 10^{-4} M ethanol as the solvent. The results were compared with that of the mitochondria treated with 10^{-4} M ethanol alone.

Determination of free radicals in the mitochondria

Higher concentrations of mitochondrial suspension was used for the determination of free radicals, because of the sensitivity of the ESR spectrometer. Reaction was started by the addition of 0.5 ml mitochondrial suspension (50–100 mg protein) to 4.5 ml of reaction medium (state 1). After 1 min, 0.5 ml of 0.2 M succinate was added (state 4). In each respiratory state, 0.5 ml of reaction mixture was took up and poured into the sample tube made of quartz and frozen in liquid nitrogen as quickly as possible. The samples were stored in liquid nitrogen and free radicals were determined within 6 hours.

In the ethanol group and the nitroglycerin group, reaction medium contained 10^{-4} M ethanol and 10^{-6} M nitroglycerin with 10^{-4} M ethanol prior to the addition of mitochondrial suspension.

For the observation of ESR signal, a JES-ME 3X ESR spectrometer (Japanese

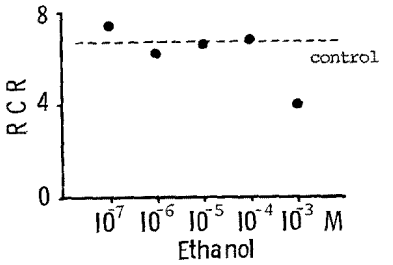

Fig. 1. Effects of ethanol on respiratory control ratio (RCR) of dog heart mitochondria. Respiratory control ratio of the mitochondria isolated from normal dog heart was 6.75±0.50. When the mitochondria were treated with ethanol for one minute prior to the addition of succinate, ethanol had no effect on the respiratory control ratio at 10^{-4} M and lower concentrations.

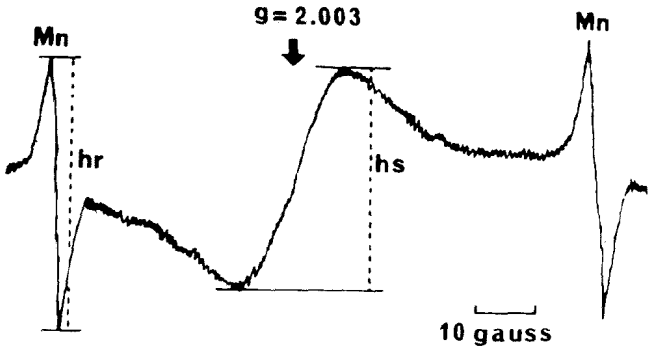

Fig. 2. ESR signal of mitochondrial preparation. ESR signal of the mitochondrial preparation was observed at g=2.003 between the third (g=1.986) and forth (g=2.026) signals of manganase chloride (Mn). ESR intensity was defined as the ratio of the maximum deflexion of the ESR signal of the sample to that of the standard signals of manganase chloride.

Electron Optics Laboratory Co.) was employed. The sample tube was inserted into the Dewar flask filled with liquid nitrogen (−196°C) and measured in the frozen state. Manganase chloride was used as the magnetic field marker and the standard substance. Conditions of ESR signal observation were as follows: 1) resonance cavity; TE 001, 2) microwave; 9410–9430 Mc, 3) power; 0.6–0.8 W, 4) modulation amplitude; 100 Kc, 5.0 Gauss, 5) sweep width; 3300±50 Gauss, 6) scan rate; 100 Gauss/min, 7) resonance time; 1.0 sec. ESR signals of mitochondrial preparation were observed at g=2.003 between the third (g=1.986) and forth (g=2.026) signals of manganase chloride. ESR intensity was defined as the ratio of the maximum deflexion of the ESR signals of samples to that of the standard signals of manganase chloride inserted in the same resonance cavity of ESR spectrometer (Fig. 2).

Statistical analysis

All data were expressed as mean±SD. Statistical analysis was made by paired t test.

Results

ESR intensity in state 1 and state 4 respiration in the 3 groups was shown in Fig. 3. In the normal control, ESR intensity increased from 0.115±0.030 per mg protein in state 1 to 0.178±0.017 in state 4. When the mitochondria were treated with 10^{-4} M ethanol, ESR intensity decreased from 0.163±0.034 in state 1 to 0.137±0.019 in state 4. When the mitochondria were treated with 10^{-6} M nitroglycerin, in spite of the presence of 10^{-4} M ethanol, ESR intensity increased from 0.124±0.023 in state 1 to 0.153±0.024 in state 4.

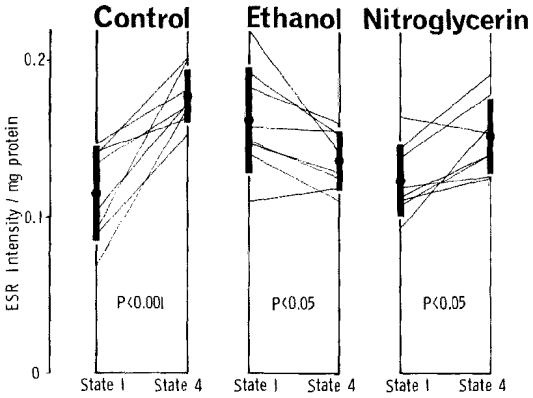

Fig. 3. ESR intensity in state 1 and state 4 respiration. Values were expressed as mean±SD. Thin lines represented the individual cases. Statistical analysis was made by paired t test.

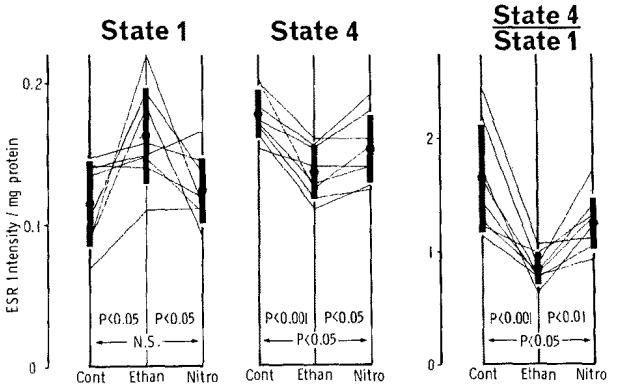

Fig. 4. Effects of ethanol and nitroglycerin on ESR intensity in state 1 and state 4 respiration, and the ratio of that in state 4 to state 1. Values were expressed as mean±SD. Statistical analysis were made by paired t test. Abbreviations: Cont=control; Ethan=ethanol; Nitro=nitroglycerin.

The effects of ethanol and nitroglycerin on the ESR intensity in state 1 and state 4 respiration were shown in Fig. 4. ESR intensity in state 1 respiration was increased by the treatment with ethanol and the effect was prevented by nitroglycerin. In contrast, ESR intensity in state 4 respiration was decreased by the treatment with ethanol and the effect was prevented by nitroglycerin.

The ratio of ESR intensity in state 4 to state 1 demonstrated more remarkably the effects of ethanol and nitroglycerin. Ethanol reduced the ratio from 1.65±0.47 in the control to 0.86±0.13 and the reduction was prevented by nitroglycerin to 1.25±0.23 (Fig. 4).

Discussion

Several approaches have been available for the assessment of mitochondrial functions. These include biochemical and histochemical determination of the activity of mitochondrial enzymes, anatomical analysis by electron microscope and determination of the efficiency of oxidative phosphorylation in the mitochondria by measuring mitochondrial respiration in state 3 and state 4.

It is well known that free radicals are produced as a result of the enzymatic redox activity in the mitochondria.[12),13)] It has been demonstrated in our previous study[14)] that free radicals arose when succinate was added to mitochondrial preparation and any free radicals could not be observed when the mitochondrial preparation was boiled at 100°C for a few seconds. The free radicals once produced decreased more rapidly under aerobic condition than under anaerobic condition. In normal heart mitochondria, free radical concentrations increased in state 4 respiration to two-fold of that in state 1, whereas they decreased in state 3 to one third of that in state 4.[15),18)] A reduction in free radical concentrations in state 4 respiration was observed in the mitochondria treated with dinitrophenol as an uncoupler and the similar changes were observed in the mitochondria isolated from ischemic dog hearts.[15),18)] In addition, a positive correlation was observed between free radical concentrations in state 4 respiration and respiratory control ratio.[16),17)]

Although the free radicals observed in this study remain not to be identified, they are supposed to be composed of flavine semiquinone, substrate free radical and fumarate-flavine-Fe^{+++}-Fe^{++}.[14)] Whatever kinds of free radicals they may be, they arise as intermediates in electron transport system and the concentrations in state 4 respiration or the ratio of that in state 4 to state 1 was proposed to be an useful index for the evaluation of mitochondrial function, such as oxidative phosphorylation.[16)]

Chronic administration of ethanol has been reported to cause the changes in oxidative phosphorylation of myocardial mitochondria as well as mitochondrial swelling and fragmentation of cristae.[21)-23)] It has been also suggested that frequent bouts of acute alcoholism might cause alterations in mitochondrial membrane permeability.[24)] In isolated ventricular muscle of frogs and cats, ethanol depressed muscle contration and shortened action potential duration at 1.5×10^{-2} M and higher concentrations.[25)] In isolated mitochondria in this study, ethanol had no effect on respiratory control ratio at 10^{-4} M and lower concentrations. In contrast, free radical formation of mitochondria was affected by 10^{-4} M ethanol. These results suggest that the measurement of free radical formation of the mitochondria can be used for the detection of

the mild changes in mitochondial function that cannot be detected by respiratory control ratio.

Nitroglycerin is the most important drug in the treatment of angina pectoris and recently it has been used for the treatment of acute myocardial infarction and congestive heart failure. The beneficial action of the drug is generally attributed to its peripheral vascular effects, that result in a reduction in preload and afterload, and work of the heart.[1)-6)]

Although metabolic effects of the drug have been investigated in several studies,[3),26)-28)] most of them were performed using whole animals or perfused hearts and the changes were attributed largely to the hemodynamic effects of the drug. According to Szekeres et al,[11)] 5×10^{-4} M nitroglycerin reduced swelling of the mitochondria and simultaneously increased respiratory control ratio and ADP/O ratio in isolated rabbit heart mitochondria, if higher concentrations of inorganic phosphate was present in the medium. They concluded that nitroglycerin was able to prevent the impairement of the efficiency of oxidative phosphorylation produced by increased ion tranport, presumably by a mitochondrial membrane-stabilizing action of nitroglycerin. In this study as well, 10^{-6} M nitroglycerin prevented the impairement of free radical formation of the mitochondria induced by ethanol.

From these results, a direct metabolic effect on myocardial mitochondria was suggested as a mechanism of the action of nitroglycerin.

References

1. Brachfeld N, Bozer J, Gorlin R: Action of nitroglycerin on the coronary circulation in normal and in mild cardiac subjects. Circulation **19**: 697, 1959
2. Gorlin R, Brachfeld N, MacLeod C, Bopp P: Effect of nitroglycerin on the coronary circulation in patients with coronary artery disease or increased left ventricular work. Circulation **19**: 705, 1959
3. Mason DT, Zelis R, Amsterdam EA: Actions of the nitrites on the peripheral circulation and myocardial oxygen consumption: significance in the relief of angina pectoris. Chest **59**: 296, 1971
4. Sonnenblick EH, Skelton CL: Myocardial energetics. Basic principles and clinical implications. New Engl J Med **285**: 668, 1971
5. Bernstein L, Friesinger GC, Lichtlen PR, Ross RS: The effect of nitroglycerin on the systemic and coronary circulation in man and dogs. Myocardial blood flow measured with Xenon 133. Circulation **33**: 107, 1966
6. Vatner SF, Pagani M, Rutherford JD, Millard RW, Manders WT: Effects of nitroglycerin on cardiac function and regional blood flow distribution in conscious dogs. Am J Physiol **234**: H244, 1978
7. Winbury MM: Redistribution of left ventricular blood flow produced by nitroglycerin. Circulat Res **28-29** (Suppl 1): 140, 1971
8. Becker LC, Fortuin NJ, Pitt B: Effect of ischemia and antianginal drugs on the distribution of radioactive microspheres in the canine left ventricle. Circulat Res **21**: 263, 1971
9. Ogawa K, Gudbjarnason S, Bing RJ: Nitroglycerin (glyceryl trinitrate) as a monoamine

oxidase inhibitor. J Pharmacol Exp Ther **155**: 449, 1967
10. Levy JV: Effect of organic nitrates on myocardial oxygen consumption in vitro. Brit J Pharmacol **38**: 743, 1970
11. Szekeres L, Vághy P, Bor P, Csete K: Possible mode of action of nitroglycerin on heart mitochondria. *in* Recent Advances in Studies on Cardiac Structure and Metabolism, Vol 11, Heart Function and Metabolism, ed by Kobayashi T, Sano T, Dhalla NS, University Park Press, Baltimore, p495, 1978
12. Commoner B, Hollocher TCJR: Free radicals in heart muscle mitochondrial particles. General characteristics and localization in the electron transport system. Proc Nat Acad Sci **46**: 405, 1960
13. Hollocher TCJR, Commoner B: Free radicals in heart muscle mitochondrial particles. Mechanism of formation. Proc Nat Acad Sci **46**: 416, 1960
14. Suzuki Y: Studies on the free radicals in myocardial mitochondria by electron spin resonance (ESR) spectrometry (Studies on experimental infarction dogs). Jpn Circulat J **39**: 683, 1975
15. Kakizawa N: Studies on free radical formation and respiratory function of heart mitochondria in experimental coronary occlusion. J Jap Coll Angiol **17**: 505, 1977
16. Suzuki Y, Yamazaki N, Ogawa K, Mizutani K, Kakizawa N, Yamamoto M, Okubo M, Yoshida M: Studies on myocardial mitochondria in failing dog hearts. Studies by electron spin resonance (ESR) spectrometry. *in* Recent Advances in Studies on Cardiac Structure and Metabolism, Vol 11, Heart Function and Metabolism, ed by Kobayashi T, Sano T, Dhalla NS, University Park Press, Baltimore, p599, 1978
17. Yamamoto M: Studies on free radical formation and respiratory function in mitochondria from experimentally-induced failing dog heart. Jpn Circulat J **42**: 1385, 1978
18. Yamazaki N, Suzuki Y, Kakizawa N, Kamikawa T: Studies on myocardial mitochondria in ischemic dog hearts by electron spin resonance (ESR) spectrometry. *in* Advances in Myocardiology, Vol 2, ed by Tajuddin M, Bhatia B, Siddiqui HH, Rona G, University Park Press, Baltimore, p315, 1980
19. Chance B, Hagihara B: Intracellular respiration. Phosphorylating and non-phosphorylating oxidation reactions. Fifth International Congress of Biochemistry, Pergamon Press, New York, p3, 1963
20. Estabrook RW: Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. Methods in Enzymology **10**: 41, 1967
21. Gvozják A, Bada V, Krutý F, Niederland TR, Gvozják J: Effect of ethanol on the metabolism of the myocardium and its relationship to development of alcoholic myocardiopathy. Cardiology **58**: 290, 1973
22. Hibbs RG, Ferrans VJ, Black WC, Weilbaecher DG, Walsh JJ, Burch GE: Alcoholic cardiomyopathy. An electron microscopic study. Am Heart J **69**: 766, 1965
23. Alexander CS: Idiopathic heart disease. II. Electron microscopic examination of myocardial biopsy specimens in alcoholic heart disease. Am J Med **41**: 229, 1966
24. Gould L: Cardiac effects of alcohol. Am Heart J **79**: 422, 1970
25. Fisher VJ, Kavaler F: The action of ethanol upon the action potential and contraction of ventricular muscle. *in* Recent Advances in Studies on Cardiac Structure and Metabolism, Vol 5, Basic Functions of Cations in Myocardial Activity, ed by Fleckenstein A, Dhalla NS, University Park Press, Baltimore, p415, 1975
26. Smith ER, Redwood DR, Maccarron WE, Epstein SE: Coronary artery occlusion in the conscious dog. Effects of alterations in arterial pressure produced by nitroglycerin, hemorrhage, and alpha-adrenergic agonists on the degree of myocardial ischemia. Circulation **47**: 51, 1973
27. Gmeiner R, Knapp E, Schwingshackl H, Dienstl F: Hypoxic, isolated rat heart. Hemodynamic and metabolic effects of nitroglycerin. *in* Recent Advances in Studies on Cardiac Structure and Metabolism, Vol 7, Biochemistry and Pharmacology of Myocardial Hypertrophy, Hypoxia, and Infarction, ed by Harris P, Bing RJ, Fleckenstein A, University Park Press, Baltimore, p405, 1976

28. Ichihara K, Abiko Y: The effect of nitroglycerin, dipyridamole, and propranolol on glycogen metabolism during coronary artery ligation in dogs. *in* Recent Advances in Studies on Cardiac Structure and Metabolism, Vol 12, Cardiac Adaptation, ed by Kobayashi T, Ito Y, Rona G, University Park Press, Baltimore, p531, 1978