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Microdialysis study of ischemia-induced hydroxyl radicals in the canine heart

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Abstract. A new experimental approach for spin-trapping of oxygen radicals in a selected region of the heart in situ is described. This approach is based on microdialysis, and it permits the detection of oxygen radicals in conditions of local ischemia and restoration of normal blood flow. Increased hydroxyl radical generation in an ischemic area of canine myocardium, as a result of 40 min local occlusion, has been studied.
Key words. Canine heart; electron paramagnetic resonance; local ischemia; microdialysis; oxygen radicals; spin-trapping.

Highly reactive free radical species, such as oxygen-derived metabolites, appear to play an essential role in a variety of pathologies, including ischemia/reperfusion-induced injury of cardiac muscle[1-4]. An understanding of the mechanism by which the active forms of oxygen participate in irreversible damage of myocardium depends upon the ability to detect and identify these short-lived free radicals. It has become possible to detect oxygen radicals generated in cardiac tissue using the method of spin-trapping[5-17]. In most cases, oxy-radical production during ischemia and reoxygenation has been studied on isolated perfused hearts or in situ animal models when only the EPR spectra of spin adducts present in perfusion medium or in coronary blood could be registered. With the development of low-frequency EPR spectrometers, it has become possible to detect radicals in whole organs and tissues[18-20], but EPR spectroscopy in vivo has strict limitations and low sensitivity. In many circumstances, it is impracticable to inject spin traps into an organism because oxygen radical spin adducts are not stable in the blood system and cells where they can be easily metabolized[21,22]. It is impossible to discriminate the contribution of different regions of myocardium to the total effect of oxy-radical production by studying the spin-trapping process in perfusion medium passing through the isolated heart or in blood from the heart working in situ.

In the present study, a new experimental approach was used to study oxygen-derived free radicals in heart working in conditions in situ. This approach is based on the canine model of regional myocardial ischemia and the method of microdialysis[23]. During such in situ experiments, the spin trap dissolved in Ringer's solution was isolated from the blood flow, passing only through a small dialysis tube implanted into the cardiac tissue. This experimental model conveniently enabled us to detect oxygen radical spin adducts formed in selected regions of canine myocardium in conditions of both local ischemia and normal blood supply.

Materials and methods

Animal preparation. Adult mongrel dogs (n = 7) of either sex weighing 10–15 kg were anesthetized with nembutal (35 mg/kg b.wt, i.p. injection). The chest was opened and animals were incubated and artificially ventilated with room air supplemented with oxygen. Left ventricular pressure (P) and its first derivative, estimating its positive (+dP/dt) and negative (−dP/dt) peaks, as well as heart rate (HR), were measured continuously using a catheter introduced into the left ventricle through the right carotid artery (fig. 1).

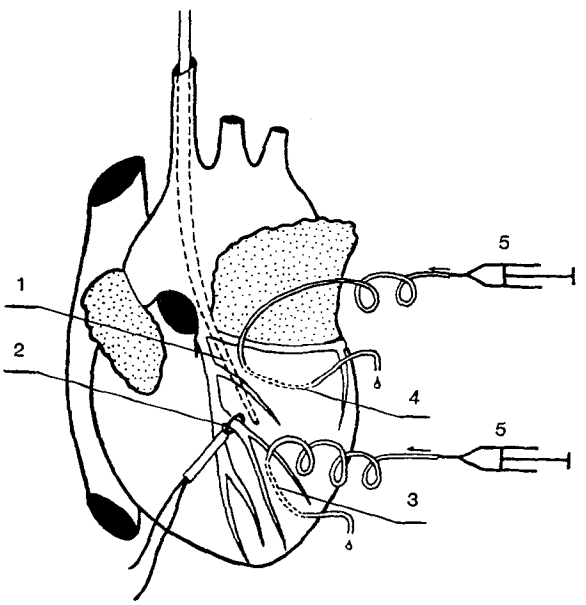

Figure 1. Scheme of experiment: 1, catheter in the left ventricle; 2, occluder; 3, dialysis tube in ischemic area; 4, dialysis tube in 'control' area; 5, syringe of perfusion pump.

Cardiac microdialysis technique. At the beginning of the experiment a temporary (10–15 s) occlusion of 1–2 small branches of anterior descending coronary artery was performed to identify the cyanotic area of the left ventricle. With the aid of a bent needle, a single dialysis fiber from this area ('ischemic' area, fig. 1), was pulled through the myocardium to a depth of about 1.5–2.0 mm. A control microdialysis probe was similarly implanted into the area of the left ventricle with a nonaffected blood supply ('control' area, fig. 1). The effective length of the dialysis fibers (0.25 mm outer diameter and 5,000 molecular weight cut-off; Cordis Dow, Belgium) was approximately 21 mm. Both ends of the fibers were inserted into inflow and outflow silicon tubes and sealed in place with cyanoacrylic glue. The inflow tubes were connected to glass syringes of a perfusion pump, the outflow tubes served for dialysate collection. After implantation the microdialysis probes were perfused at a rate of 3 µl/min with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$)[23] with added 100 mM DMPO (5,5-dimethyl-1-pyrroline-N-oxide; Aldrich, USA). Prior to the experiment, Ringer's solution was degassed under vacuum, and the microdialysis probes were washed with degassed solution for 40 min.

Experimental protocol. Initially the heart was allowed to work for 30 min under conditions of normal blood supply. Then local ischemia was induced by a 40 min occlusion of the 1–2 small branches of the coronary artery. Next the ligature was removed, and reperfusion occurred in the 'ischemic' area of myocardium. From the beginning of the experiment the dialysate samples from each fiber were collected every 10 min (or 20 min) in microtubes, and immediately frozen in liquid nitrogen.

EPR measurements. The EPR spectra were recorded at room temperature on a E-109E X-band spectrometer (Varian, USA), with a modulation frequency of 100 kHz, modulation amplitude of 0.1 mT and microwave power of 10 mW. The frozen samples of dialysate were thawed just before their EPR spectra were obtained. The control experiments showed that when samples containing oxygen radical spin adducts were frozen and stored at the temperature of liquid nitrogen, no significant changes in their EPR spectra occurred during the measurements. DMPO spin adduct concentrations in samples were determined by comparing EPR spectra of the adducts with the EPR spectrum of a stable nitroxide radical TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy; Aldrich, USA).

Statistical analysis. Data shown in the table and figure 2 were expressed as mean ± standard deviation.

Results and discussion

We studied the effects of local ischemia and postischemic restoration of normal blood supply on the physiological characteristics of heart function and DMPO spin adduct content of dialysate passed through selected regions ('control' and 'ischemic' areas, fig. 1) of canine myocardium. The table presents the values of physiological parameters of cardiac muscle which were measured at different stages of the experiment. As shown by this table, neither elimination of blood flow in one of the areas of the coronary bed nor subsequent restoration of it induced any significant changes in the hemodynamic variables of canine heart working in situ. This demonstrates that in our experiments the effect of local ischemia on global cardiac function was insignificant. Therefore, the physiological conditions in the areas of myocardium with normal blood supply, including the 'control' area (fig. 1), could be regarded as nonaffected ones throughout the experiment. In our previous study[23], by monitoring the content of adenine nucleotide breakdown products in cardiac dialysate, we have differentiated the ischemia/reperfusion-induced injury of canine myocardium.

To detect the oxygen radical spin adducts formed in both 'control' and 'ischemic' areas of canine myocardium (see fig. 1), we recorded the EPR spectra of collected dialysate samples. The EPR spectra of all measured samples consisted predominantly of four equidistant components with a ratio of intensities

Values of the main physiological characteristics of hearts

Stage of experiment	P (mm Hg)	+dP/dt (mm Hg/s)	−dP/dt (mm Hg/s)	HR (beats/min)
Initial perfusion*	93 ± 8	3658 ± 408	2461 ± 378	150 ± 11
40 min of ischemia	93 ± 11	3491 ± 591	2660 ± 403	145 ± 7
80 min of reperfusion	99 ± 7	3890 ± 530	3026 ± 475	138 ± 8

*Values obtained just before beginning of ischemia.

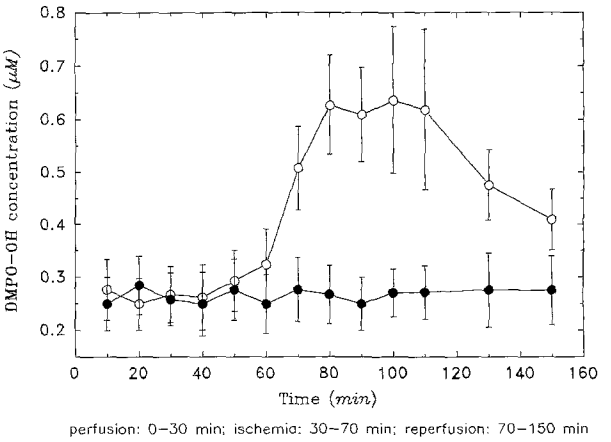

Figure 2. Values of concentration of the hydroxyl radical spin adduct of DMPO in dialysate samples, corresponding to ischemic (graph with open circles) and 'control' (graph with filled circles) areas of mycardium and registered at the different stages of experiment.

1:2:2:1. Such a spectrum indicates the presence of hydroxyl radical spin adduct in the dialysis medium. In postischemic reperfused heart, the superoxide anion and hydrogen peroxide are throught to be the precursors of hydroxyl radicals, and they are believed to originate from cardiac mitochondria, vascular endothelial cells and infiltrating neutrophils[3,4]. The EPR spectra of DMPO spin adducts allow us to identify oxygen-derived free radicals, but in our experiments there were no recorded components in any spectra that might be attributed to the products of DMPO reacting with superoxide radical. However, DMPO-OOH has been shown to decompose to the hydroxyl spin adduct of DMPO[24,25], resulting in uncertainty as to whether the detection of DMPO-OH in canine myocardium during local ischemia and reperfusion resulted from the presence of superoxide and/or hydroxyl radicals.

Figure 2 presents the results characterizing the content of DMPO-OH in the samples of dialysate that had passed through both the 'control' and 'ischemic' regions of heart. In the samples from the 'ischemic' area, 20–30 min of absence of normal blood supply induces a stepwise increase in the intensity of hydroxyl radical spin adduct signal (fig. 2, graph with open circles). After 40 min of reperfusion, there is a significant decrease in the DMPO-OH concentration in dialysate. These data indicate that similar changes occurred in the number of oxygen-derived free radical species which could react with DMPO in the 'ischemic' area of myocardium. At the same time, the concentration of DMPO spin adducts in the dialysate samples from the 'control' area failed to show any change throughout all spin-trapping experiments (fig. 2, graph with filled circles).

Thus, the experimental model of the canine heart working in conditions in situ enabled us to demonstrate an increased rate of oxygen radical production in the selected area of left ventricle during local ischemia and subsequent restoration of normal blood flow. Commonly, investigators have supposed (see refs 5–17) that oxygen free radicals are not produced in ischemic myocardium in conditions of complete absence or very low pressure of oxygen. This seems to be the case in experiments using isolated hearts during global, non-flow ischemia. However, in our experiments, regional myocardial ischemia was used, and it is logical to explain the appearance of oxy-radical species after 30 min of regional ischemia by the existence of continuous collateral circulation[26] (3–15% of the normal resting blood flow) and the initiation of irreversible changes in cardiac tissue. Furthermore, in experiments using the microdialysis technique, it is of critical importance that the formation and, consequently, the spin-trapping of short-lived active oxygen intermediates may occur both in the myocardial tissue and in the perfusion medium itself. On passing the Ringer's solution with DMPO through the dialysis fiber, the spin trap molecules can penetrate through its semipermeable walls into the heart, and the molecules of spin adduct can move back into the dialysis fiber. In addition, some low molecular weight substances that are thought to be released from myocytes during ischemia (ref. 4) may also be present in the dialysis fiber. If this is the case, at least part of the oxygen radicals that can be spin-trapped in microdialysis experiments are generated not in the cardiac muscle but in the dialysis medium.

1 Hess, M. L., and Manson, N. H., J. molec. cell. Card. *16* (1984) 969.
2 McCord, J. M., New Engl. J. Med. *312* (1985) 159.
3 Flaherty, J. T., and Weisfeldt, M. L., Free Rad. Biol. Med. *5* (1988) 409.
4 Oxygen Radicals in Pathophysiology of Heart Disease. Ed. P. K. Singal. Kluwer Academic Publishers, Boston-Dordrecht-Lancaster 1988.
5 Kramer, J. H., Arroyo, C. M., Dickens, B. F., and Weglicki, W. B., Free Rad. Res. Commun. *3* (1987) 153.
6 Blasig, I. E., Ebert, B., Wallukat, G., and Loewe, H., Free Rad. Res. Commun. *6* (1989) 303.
7 Bolli, R., Jeroudi, M. O., Patel, B. S., Aruoma, O. I., Halliwell, B., Lai, E. K., and McCay, P. B., Circulation Res. *65* (1989) 607.
8 Culcasi, M., Pietri, S., and Cozzone, P. J., Biochem. biophys. Res. Commun. *15* (1989) 1274.
9 Politi, P. M., Rajagopalan, S., and Sinha, B. K., Biochim. biophys. Acta *992* (1989) 341.
10 Zweier, J. L., Kuppusamy, P., Williams, R., Rayburn, B. K., Smith, P., Weisfeldt, M. L., and Flaherty, J. T., J. biol. Chem. *264* (1989) 18890.
11 Kuzuya, T., Hoshida, S., Kim, Y., Nishida, M., Fuji, H., Kitabatake, A., Tada, M., and Kamada, T., Circulation Res. *66* (1990) 1160.
12 Shuter, S. L., Davies, M. J., Garlick, P. B., Hearse, D. J., and Slater, T. F., Free Rad. Res. Commun. *9* (1990) 223.
13 Tosaki, A., and Braquet, P., Am. Heart J. *120* (1990) 819.
14 Blasig, I. E., Steinschneider, A. Y., Lakomkin, V. L., Ledenev, A. N., Korchazhkina, O. V., and Ruuge, E. K., FEBS Lett. *267* (1990) 29.
15 Coghlan, J. B., Flitter, W. D., Holley, A. E., Norell, M., Mitchell, A. G., Ilsley, C. D., and Slater, T. F., Free Rad. Res. Commun. *14* (1991) 409.
16 Nohl, H., Stolze, K., Napetschnig, S., and Ishikawa, T., Free Rad. Biol. Med. *11* (1991) 581.
17 Packer, L., Valenza, M., Serbinova, E., Starke-Reed, P., Frost, K., and Kagan, V., Archs Biochem. Biophys. *288* (1991) 533.
18 Rosen, G. M., Halpern, H. J., Brunsting, L. A., Speneer, D. P., Strauss, K. E., Bowman, M. K., and Wechsler, A. S., Proc. natl Acad. Sci. USA *85* (1988) 7772.
19 Zweier, J. L., Thompson-Gorman, S., and Kuppusamy, P., J. Bioenerg. Biomembr. *23* (1991) 855.
20 Glockner, J. F., and Swartz, H. M., Adv. expl Med. Biol. *317* (1992) 229.
21 Samuni, A., Samuni, A., and Swartz, H. M., Free Rad. Biol. Med. *6* (1989) 179.
22 Rashba-Step, J., Turro, N. J., and Cederbaum, A. I., Archs Biochem. Biophys. *300* (1993) 391.
23 Kuzmin, A. I., Tskitishvili, O. V., Serebryakova, L. I., Saprygina, T. V., Kapel'ko, V. I., and Medvedev, O. S., J. cardiovasc. Pharmac. *20* (1992) 961.
24 Finkelstein, E., and Rosen, G. M., Adv. Free Rad. Biol. Med. *1* (1985) 345.
25 Cohen, M. S., Britigan, B. E., Hasset, D. J., and Rosen, G. M., Free Rad. Biol. Med. *4* (1988) 81.
26 Koke, J. R., and Bittar, N., Cardiovasc. Res. *12* (1978) 309.