Formation of Hydroxyl Radicals during Myocardial Reperfusion after Experimental Ischemia of Different Duration

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The intensity of hydroxyl radical ($OH^{\bullet}$) formation in the myocardium during reperfusion after ischemia of different duration was evaluated using microdialysis with sodium salicylate. 2,3-Dihydroxybenzoic acid, a product of $OH^{\bullet}$ trapping by salicylic acid, was used as a marker of $OH^{\bullet}$ generation in the postischemic myocardium. Experiments were performed on open-chest anesthetized and jet-ventilated Wistar rats. The concentrations of 2,3-dihydroxybenzoic acid in the dialysate were measured by high performance liquid chromatography (HPLC) with electrochemical detection. Experiments showed that the intensity and duration of free oxygen radical generation during reperfusion after 30-min ischemia far surpassed those observed after 20-min ischemia.

Key Words: *free oxygen radicals*; *salicylate*; *myocardium*; *ischemia/reperfusion*; *rats*

Enhanced generation of free oxygen radicals (FOR) plays a major role in myocardial injury during postischemic reperfusion [3,6]. In most experimental studies, the content of FOR are measured in the perfusate (for isolated hearts) [11,12] or in coronary blood *in vivo* [1,9] using a spin-trap technique. However, local events occurring in the focus of ischemia or reperfusion could not be assessed by this method.

Immediately after the start of reperfusion oxygen enhanced production of superoxide anion $O_2^{\bullet -}$, which reacts with iron ions and forms short-living and highly reactive hydroxyl radical $OH^{\bullet}$ [2]. Chemical approach based on the reaction of $OH^{\bullet}$ with salicylic acid is a sensitive, selective, and inexpensive methods for $OH^{\cdot}$ detection [7,8,10]. A combination with microdialysis technique this method allows a long-term continuous *in vivo* monitoring $OH^{\bullet}$ level directly in the ischemic/reperfusion focus, even when blood supply to the heart is arrested. Such an approach is very informative, but it is rarely used for *in vivo* studying of the role of free oxygen radicals in postischemic myocardial injury [8,13].

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In this work we evaluated generation of $OH^{\bullet}$ in the myocardium during reperfusion after different periods of ischemia using a sodium salicylate microdialysis technique. 2,3-Dihydroxybenzoic (2,3-DHBA) produced in the reaction of $OH^{\bullet}$ with salicylic acid was used as the marker of $OH^{\bullet}$ generation in the myocardium.

MATERIALS AND METHODS

Experiments were carried out on open-chest jet-ventilated male Wistar rats weighing 300-400 g narcotized with ketamine (10 mg/kg, intraperitoneally). Heart rate and blood pressure (BP, through a catheter in the femoral artery) were recorded continuously throughout the experiment. Myocardial ischemia was modeled by ligation of the descending branch of the left coronary artery immediately below the left auricle. A microdialysis fiber (outer diameter 0.3 mm, size of passing particles ≤5000 D) was implanted into the left ventricular myocardium so that it passed through the ischemic region detected by a short-term occlusion of the descending branch of the left coronary artery (Fig. 1). The length of dialysis fiber implanted into the myocardium varied from 5 to 8 mm, therefore the original data for each animal were standardized to a

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dialyser length of 6 mm. The implanted dialyser was perfused for 1 h with standard Ringer solution containing 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$ (pH 7.4) at a flow rate of 3 μl/min and then with salicylate-containing Ringer solution (1 μM/ml, pH 7.4) until stabilization of the 2,3-DHBA level. Three baseline dialysate samples were collected. In one group of rats (n=6) ischemia lasted for 20 min and in other group (n=6) for 30 min; reperfusion was performed for 60 min in both experimental groups. Ten- and 5-min fractions of the dialysate were collected.

Freshly collected dialysate aliquots were analyzed by HPLC with electrochemical detection on a HR-80 column (80×4.6 mm, 3 μ sorbent particles size) under isocratic conditions at 1 ml/min elution rate. Mobile phase contained 35 mM KH_2PO_4; 30 mM citric acid, 2 mM Na_2EDTA; 100 mM sodium octanesulphonate; and 13% methanol (pH 2.3). The sensitivity threshold for 2,3-DHBA was 5 pg at a 3:1 signal-noise ratio. In special experimental series, the sensitivity and adequacy of the method were evaluated.

The experimental data were statistically analyzed by Student's t test. The results are presented as M±SEM.

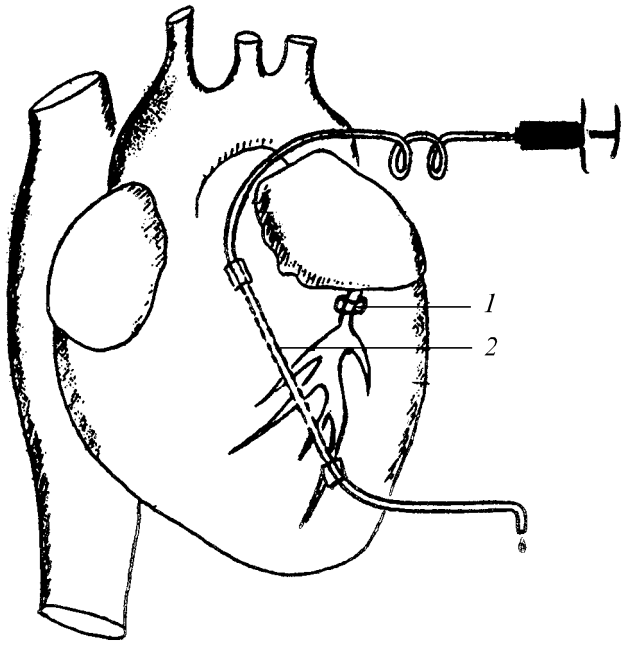

Fig. 1. Ligation of the descending branch of the left coronary artery (*1*) and dialyzer (*2*) implanted into the left ventricular myocardium.

RESULTS

The adequacy and sensitivity of the method were verified by stability of 2,3-DHBA level in the dialysate for 2.5 hour without stimulation and its changes in response to addition of 0.1 M KCl into the perfusate. It was demonstrated that $OH^{\bullet}$ generation in the myocardium rapidly and significantly increased in the presence of K^+ excess and decreased after KCl removal [8]. The stability of 2,3-DHBA baseline level required checking because sodium salicylate can be easily oxidized in contact with metals or upon light exposure, which led to overestimating of 2,3-DHBA concentration, especially by the end of the experiment. A typical feature is a sharp increase in 2,3-DHBA content in the dialysate after addition of KCl in high concentrations (Fig. 2, curve *2*), the baseline level being stable (curve *1*). It should be noted that the baseline level of 2,3-DHBA in the dialysate is determined by the presence of 2,3-DHBA admixture in sodium salicylate and by $OH^{\bullet}$ generation in tissues. In our experiments, the baseline levels of 2,3-DHBA in the dialysate did not exceed those in salicylate-containing perfusion solution, while their absolute values (6-10 pM/ml) were 3-5-fold lower than in published reports (30 pM/ml). This suggests that our modification of the method [8] is more sensitivity to changes in 2,3-DHBA content in tissues.

In animals subjected to 20-min and 30-min ischemia, the initial values of BP (108±9 and 102±9 mm Hg) and heart rate (269±14 and 276±22 bpm) were similar.

In both groups, 2,3-DHBA level remained unchanged during the first 20 min of ischemia, but after this term the concentration of 2,3-DHBA in the dialysate significantly increased (data not shown).

During reperfusion the concentration of 2,3-DHBA in the dialysate increased in all rats (Fig. 3): after 20-ischemia, the maximum increase (by 42± 16%, p<0.05) was reached during the first 10 min of reperfusion, while after 30-min ischemia, this parameter peaked between 15 and 20 min of reperfusion (by 114.5±18%

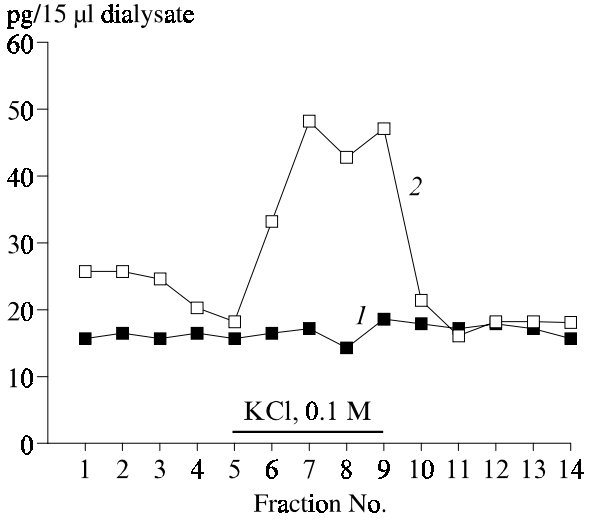

Fig. 2. Concentration of 2,3-DHBA in myocardial dialysate during perfusion of the dialyzer with Ringer solution containing sodium salicylate (1 μM/ml, pH 7.4; *1*) and during temporary perfusion with Ringer solution containing sodium salicylate and 0.1 M KCl (*2*).

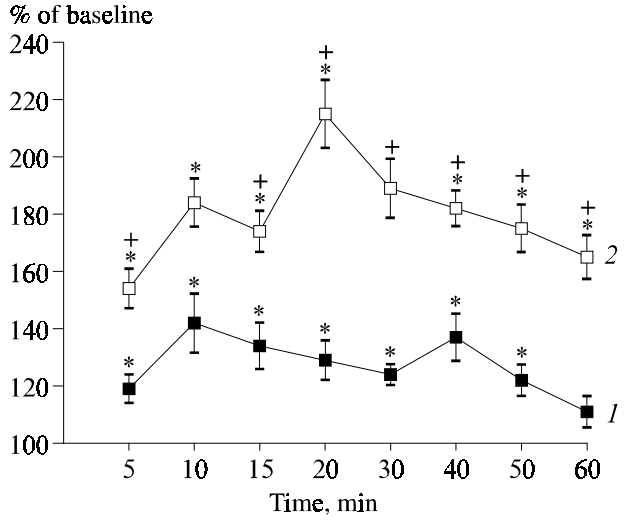

Fig. 3. Content of 2,3-DHBA in myocardial dialysate during reperfusion after 20-min (*1*) and 30-min ischemia (*2*). $p<0.05$) *compared to the baseline; +compared to 20-min ischemia.

above the baseline, $p<0.001$). After 60-min reperfusion, the concentration of 2,3-DHBA in the dialysate after 30-min ischemia remained above the baseline ($p=0.001$), while after 20-min ischemia this difference was insignificant. The integral index of $OH^{\bullet}$ generation was calculated as the area under the 2,3-DHBA concentration curve. In rats subjected to 20- and 30-min ischemia the respective values were 104.50±20.13 and 301.90±114.94 pM×h/ml ($p=0.01$).

Thus, FOR generation during reperfusion after a 30-min ischemia was more pronounced (peak value and duration) than after 20-min ischemia. One of the major ways of $O_2^{\bullet-}$ production is xanthine oxidase reaction, with xanthine and hypoxanthine acting as the substrates [6]. It was recently established that under similar experimental conditions, prolongation of regional ischemic episodes was associated with progressive accumulation of ATP catabolites (primarily xanthine and hypoxanthine). Moreover, these substances are more slowly removed during reperfusion because of more severe microcirculatory disturbances after a longer period of ischemia [4]. In other words, prolonged ischemia facilitates $O_2^{\bullet-}$ generation during subsequent reperfusion.

Thus, the data obtained using highly-sensitive salicylate technique suggest that increasing the duration of ischemic episode enhances $OH^{\bullet}$ generation increasing both its peak concentrations and duration of this process. The method of evaluation of $OH^{\bullet}$ level by the product of its reaction with salicylic acid is characterized by high sensitivity allowing to detect changes in 2,3-DHBA concentrations with an accuracy of 2-3 pM/ml. The method is simple and inexpensive, and possesses advantage over the spin-trap method.

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