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Selective Antioxidant Enzymes during Ischemia/Reperfusion in Myocardial Infarction

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Summary

The study of ischemia/reperfusion injury included 25 patients in the acute phase of myocardial infarction (19 perfused, 6 remained non-reperfused as evaluated according to the time course of creatine kinase and CK-MB isoenzyme activity) and a control group (21 blood donors). Plasma level of malondialdehyde was followed as a marker of oxidative stress. Shortly after reperfusion (within 90 min), a transient increase of malondialdehyde concentration was detected. The return to the baseline level was achieved 6 h after the onset of therapy. The activity of a free radical scavenger enzyme, plasma glutathione peroxidase (GPx), reached its maximum 90 min after the onset of treatment and returned to the initial value after 18 h. The specificity of the GPx response was confirmed by comparing with both non-reperfused patients and the control group, where no significant increase was detected. The erythrocyte Cu,Zn-superoxide dismutase (SOD) did not exhibit significant changes during the interval studied in perfused patients, probably due to the stability of erythrocyte metabolism. In non-reperfused patients, a decrease of SOD was found during prolonged hypoxia. These results help to elucidate the mechanisms of fast activation of plasma antioxidant system during the reperfusion after myocardial infarction.

Key words

Superoxide dismutase • Glutathione peroxidase • Malondialdehyde • Myocardial infarction • Oxidative stress

Introduction

Thrombolysis and primary angioplasty have become the classical procedures for treating acute myocardial infarction (AMI). The development of these techniques, which restore the flow of oxygenated blood to ischemic myocardial tissue, has led to major advances in the treatment of AMI. However, it has been recognized that the process of myocardial reperfusion may itself lead to a number of adverse consequences, including myocardial stunning, reperfusion arrhythmia or an increase in infarct size (Kerr *et al.* 1996). The hypoxia of myocardial tissue occurring during ischemia has various negative effects, including a release of proteolytic

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enzymes and damage of respiratory chain. The activity of enzyme antioxidants during ischemia remains low or even decreases (Dhaliwal *et al.* 1991). Complete or partial restoration of oxygenated blood flow during reperfusion leads to a sudden massive increase in oxygen concentration, which results in an imbalance of oxidative/antioxidative processes. The excess of oxygen leads to the production of reactive oxygen species, which may initiate lipid peroxidation in cell membranes, damage membrane proteins or cause DNA fragmentation. These processes may result in a loss of heart contractile function and lead to severe myocardial cell damage (Chen *et al.* 1995).

Direct evidence of increased free radical production at the early stage after recanalisation in a patient undergoing primary coronary angioplasty for AMI was obtained by electron paramagnetic resonance spectroscopy (Grech *et al.* 1993). As the half-life of oxygen free radicals (OFR) is very short, their formation has been usually measured indirectly – by assaying the myocardial content of malondialdehyde (MDA), a product of oxidative degradation of polyunsaturated fatty acids with more than two double bonds. Under normal physiological conditions, the level of OFR is controlled by antioxidant enzyme system, namely by the free radical scavenger enzyme superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Lin *et al.* 1997). In addition, there is also a large number of natural or synthetic antioxidant compounds, which have the ability to attenuate the oxidative damage either by scavenging highly destructive free radical species or by inhibiting lipid peroxidation in biological membranes (e.g. vitamin C, E, β-carotene, flavonoids) (Hess and Kukreja 1998).

A fine balance between oxygen free radicals and a variety of endogenous antioxidants is crucial for avoiding the myocardial injury. The study of antioxidant capacity under pathological conditions may lead to better understanding of the role of individual components of the antioxidant enzyme system in myocardial protection. From these reasons the dynamics of free radical formation in reperfused and non-reperfused patients was correlated with the level of key antioxidant enzymes in our study.

Methods

Twenty-five patients (20 men, 5 women) in the acute phase of myocardial infarction and 21 control subjects (17 men, 4 women) were studied (Table 1). The patients fulfilled the following criteria:

1. the first myocardial infarction (chest pain of more than 20 min duration, electrocardiographic changes consistent with AMI: pathological Q or at least 2 mm of ST segment elevation in two precordial or two inferior leads)
2. admission within 6 h after the onset of AMI.

Table 1. Group characterization

	Acute myocardial infarction	**Control group**
No. of patients	n = 25	n = 21
Age	61.3±9.6	55.5±4.1
Male/female	20/5	17/4
Body mass index [kg.m^{-2}]	27.1±3.3	22.6±4.9
Positive family history [%]	28.0	33.3
Total cholesterol [mmol.l^{-1}]	6.0±1.0	5.9±1.1
Index athero according to Klimov	4.2±1.5	3.9±1.3
Diabetes mellitus [%]	24.0*	4.8
Hypertension [%]	56.0***	4.8
Cigarette smoking [%]	52.0*	38.0
Ferritin [μg. l^{-1}]	171±124.3***	54.1±42.2
Fibrinogen [g.l^{-1}]	2.9±0.7***	1.5±0.2

Data are expressed as means ± S.D., statistical significance is compared to controls:
*$p< 0.05$, ** $p< 0.01$, *** $p<0.001$*

Twenty-one patients were treated by thrombolytic therapy (streptokinase, Streptase-Behringwerke Co., Germany, 1.5 MIU/1 h), 4 patients could not undergo this treatment due to the contraindications (enhanced risk of bleeding). Other treatment was given when necessary. The control group included 21 blood donors of the similar age and mixed gender, with no history of cardiovascular disease. Written informed consent was obtained from all participants before the experimental protocol was initiated and the study was approved by Hospital Committee on Human Research.

Peripheral venous blood samples were obtained from each patient immediately before starting the therapy and after 1.5 h, 3 h, 6 h, 12 h, 24 h and 48 h. Samples were drawn into plastic tubes with heparin (Vacuette no. 456083, Greiner Labortechnik Co., Austria) for SOD and GPx estimation, and into plastic tubes with gel (Vacuette no. 455071, Greiner Labortechnik Co., Austria) for MDA. Freshly frozen serum samples for MDA analysis were stored at –70 °C. MDA was estimated according to Hendrix and Assman (1990) by the thiobarbituric acid (TBA) test. To achieve high specificity, the absorbance of the MDA-TBA complex was measured at three wavelengths (485, 532 and 560 nm) and the absorbance correction was calculated by Allen's equation (Hendrix and Assman 1990).

Activity of SOD was measured in washed erythrocytes (fourfold) after their lysis with the set RANSOD (Randox Co., Great Britain) using photometer Vitatron ISP (Vital Scientific, The Netherlands). Activity of GPx was estimated in the whole blood with the set RANSEL (Randox Co., Great Britain) using the same device as mentioned above.

Ferritin content was determined using the kit Spectria Ferritin [I^{125}] Coated Tube Immunoradiometric Assay (Orion Diagnostica Co., Finland) on a biochemical analyser Stratec SR 300 (Stratec Electronic GmbH, Germany). Other biochemical parameters were measured by a standard procedure on an automatic analyser Dimension AR (Dade Co., USA): cholesterol using the kit Cholesterol Liquid (Dialab Co., Austria), Creatine kinase using the kit Creatine kinase (Dade Co., USA), CK-MB using the kit Creatine kinase MB isoenzyme (Dade Co., USA). Fibrinogen concentrations were measured by enzymatic turbidimetric method using proteolytic enzyme from snake toxin E.C. 3.4.21.2. (kit Fibrinogen ET, Bio Media Co., CR) on the multichannel photometer Labsystems FP-901 (Labsystems O.Y., Finland).

Reperfusion and the extent of myocardial injury were evaluated indirectly by measuring the time course of

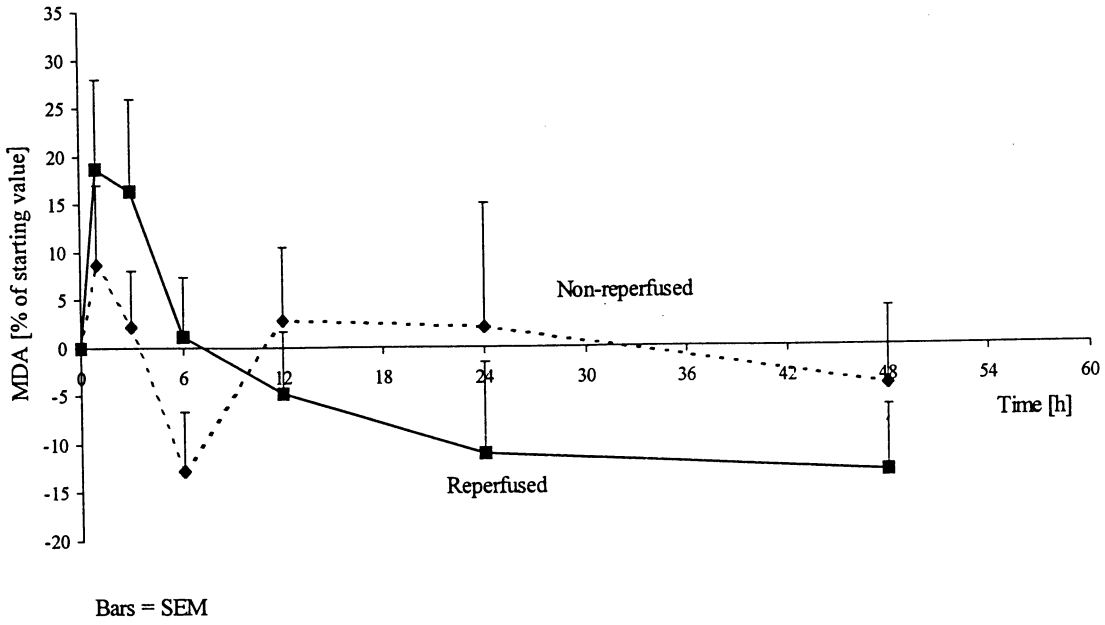

Fig 1. *The dynamics of plasma MDA level in reperfused and non-reperfused patients with acute myocardial infarction.*

creatine kinase (CK) and CK-MB isoenzyme (CK-MB) activity. Data are presented as the mean value of the difference from the starting level (in %) ± standard error of the mean (S.E.M.). The statistical significance of the differences during the observations was evaluated by the ANOVA 2P test. Differences between the control subjects and patients were determined by Student's t-test. $P<0.05$ values were considered statistically significant.

Results

Twenty-five patients with AMI were included into this study. Successful reperfusion occurred in 19 patients, i.e. 76 %. Maximum of CK and CK-MB activity was reached by 12 h after the administration of thrombolytic therapy, which is in accordance with Zabel *et al.* (1993). In these patients, necrosis of myocardium was confirmed by a significant increase in CK, the peak of which was 51.0±36.8 $\mu kat.l^{-1}$. Non-reperfused patients exhibited the CK peak later than 12 h after the onset of the therapy (47.4±29.6 $\mu kat.l^{-1}$). Comparison of the patients with AMI and the control group is given in the Table 1.

In reperfused patients the transient increase of MDA level ($p<0.05$) was found shortly after therapy initiation (Fig. 1). Maximum of MDA level was achieved within 1.5 h (rise from the basal level 1.04±0.41 μM to 1.18±0.54 μM). MDA concentration returned to basal level by 6 h. Subsequently, the MDA level decreased even below the initial value. MDA concentration in non-reperfused patients with a coronary artery occlusion did not exhibit statistically significant differences during the time of observation (Fig. 1).

The time dependence of the activity of Cu,Zn-SOD, one of the main components of the antioxidant system, is shown in Figure 2. The SOD activity in reperfused patients did not change significantly during the time period studied. This may be due to the fact that erythrocyte SOD was measured, which need not necessarily respond to the changes in the blood free radical concentration to the same extent as the plasma isoenzyme. In non-reperfused patients, a significant decrease of SOD activity ($p<0.05$) was detected in the first 12 h. This may be related to the prolonged hypoxia.

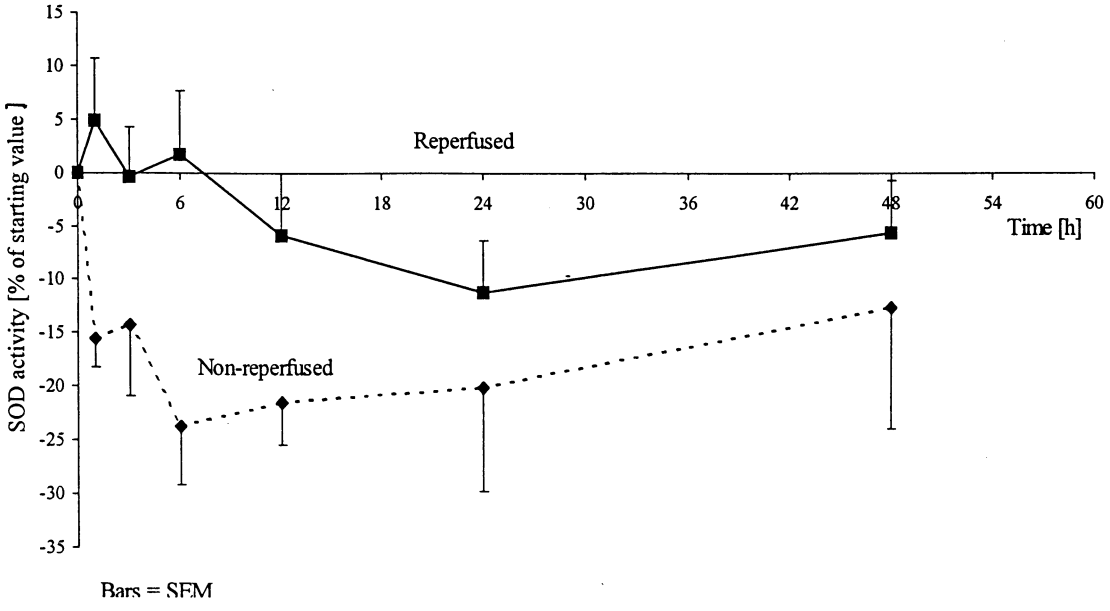

Fig 2. *The dynamics of SOD activity in erythrocytes in reperfused and non-reperfused patients with acute myocardial infarction.*

The initial level of SOD activity in patients with AMI was not significantly different the SOD activity level in control group (1008.3±197.5 U/g Hb vs. 905.3±144.0 U/g Hb, $p>0.05$).

The dynamics of glutathione peroxidase (GPx) activity level in whole blood is shown in Figure 3. Reperfusion was accompanied by temporary significant increase in GPx activity ($p<0.05$). After approximately 18 h, the enzyme activity dropped to the initial value. The enzyme activity did not significantly change in non-reperfused patients in the time period studied. When comparing the initial GPx level in AMI patients to the control group, a significant difference was found between these two groups (35.0±10.1 U/g Hb vs 41.9±10.3 U/g Hb, $p<0.05$).

Evaluation of the risk factor levels in patients with AMI and the control group revealed most significant differences in the plasma concentration of ferritin and fibrinogen and the occurrence of hypertension (Table 1). The occurrence of AMI also positively correlated with cigarette smoking and to a lesser extent with the incidence of diabetes mellitus.

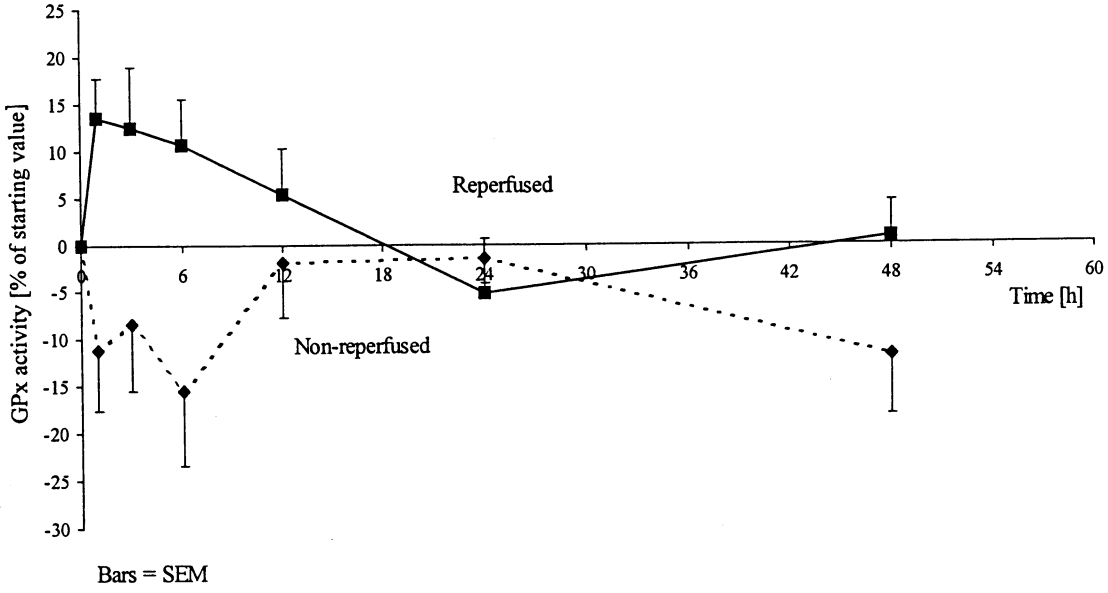

Fig 3. *The dynamics of GPx activity in whole blood in reperfused and non-reperfused patients with acute myocardial infarction.*

Discussion

While oxygen free radical (OFR) generation as well as lipid peroxidation and isomerisation remain at a low level during ischemia (Davies *et al.* 1990), early coronary recanalisation, i.e. a sudden massive supply of oxygen to previously hypoxic tissue, leads to an unbalanced burst of oxygen free radical production (Hudson 1994, Hess and Kukreja 1998, Ferrari *et al.* 1998). Subsequently, reperfusion injury, manifested by reperfusion arrhythmia, myocardial stunning or an increase in infarct size may occur (Young *et al.* 1993, Maxwell and Lip 1997).

As free radicals are difficult to estimate directly *in vivo,* because of their high reactivity and short half-life, the level of a stable product of lipid peroxidation, malondialdehyde, was used as a measure of OFR generation in this study. MDA has become the most widely used marker of free radical activity (Davies *et al.* 1990, Young *et al.* 1993, Chen *et al.* 1995, Horwitz *et al.* 1999). When estimating the MDA concentration by fluorometry, we tried to minimize the potential interference of other fluorescent substances by calculating the actual MDA level from three different wavelengths. Our results on the transient increase of MDA concentration after reperfusion confirm the hypothesis on the burst of reactive oxygen species during

the first few minutes of reperfusion after myocardial ischemia (Horwitz *et al.* 1999). In accordance with Davies *et al.* (1990), Young *et al.* (1993) and Horwitz *et al.* (1999), we found an elevation of plasma MDA concentration early after artery recanalisation. A correlation of this increase with the influx of oxygenated blood was confirmed by a comparison with the situation in non-reperfused patients, where such an increase was not found. Subsequent decrease of MDA level, which even dropped below the initial level after 6 h, may be caused by the activation of antioxidant mechanisms, especially of the enzymes SOD, catalase and GPx.

To evaluate the ability of the organism to counterbalance the oxidative stress caused by sudden excess of OFR (detected by the peak of plasma MDA) after reperfusion, the activity of two components of antioxidant defense system – SOD and GPx – was followed. SOD is involved in the primary mechanism for clearance of superoxide anions. It catalyses dismutation of superoxide anions to hydrogen peroxide and molecular oxygen. GPx, together with catalase (which is, however, present in the heart in very low concentrations) eliminate hydrogen peroxide as well as toxic hydroperoxides of unsaturated fatty acids (Gutteridge 1995).

Two distinct forms of SOD were characterized, the Cu,Zn-form present in the cytosol and the Mn-form present in mitochondria (Ferrari *et al.* 1998). In our experiments, Cu,Zn-SOD activity was measured in erythrocytes. We did not find a significant increase of Cu,Zn-SOD activity in reperfused patients, which would be parallel to the maximum of MDA found shortly after therapy initiation. The decrease of SOD activity found in non-reperfused patients during prolonged hypoxia might be caused by both substrate shortage and enzyme damage. Our results are in accordance with the findings of Tomoda *et al.* (1996), who detected increased values of Mn-SOD, but not of the Cu,Zn-form, after a longer time period (108 h after the therapy initiation). Elevated level of Mn-SOD was found to correlate with the increase of MDA plasma concentration and neutrophil activation (Simovic *et al.* 1997). Mn-SOD was effective in the protection of cultured myocytes against hypoxia/ reoxygenation injury (Qian *et al.* 1997) and has recently been found to play an important role in cardioprotection against ischemia/reperfusion injury in rats (Yamashita *et al.* 1999, Dhalla *et al.* 1999). Contrarily, a significant increase in the activity of Cu,Zn-SOD, but not of the Mn-form, was detected in porcine myocardial tissue with hypertrophic cardiomyopathy (Lin *et al.* 1997). It seems that erythrocyte enzymes, probably due to the stable erythrocyte metabolism (Lafont *et al.* 1996), may be involved in the long-term adaptation to pathological processes. Plasma Mn-SOD may represent a readily mobilizable form, which contributes to the buffering of a sudden oxidative stress. This hypothesis is supported by a comparison of our results on plasma GPx with the report on erythrocyte GPx, which did not respond to the oxidative stress by elevated activity (Lafont *et al.* 1996). The activity of plasma enzyme rapidly followed the elevation of a free radical formation. The specificity of this reaction was confirmed by comparing with both non-reperfused patients and the control group. An increase in plasma GPx after reperfusion was also reported by Pucheu *et al.* (1995).

Our results demonstrate that basal level of GPx was significantly lower in patients with AMI in comparison with the control group. The lower GPx level in patients before recanalisation might be the consequence of the enzyme damage during ischemic conditions. However, it cannot be excluded that the lower activity of GPx might have occurred in patients even before AMI manifestation and thus had contributed to the decrease of the defense system. This could lower the scavenging of OFR, which resulted in enhanced atherogenesis.

Being aware that the susceptibility towards the AMI represents a complex issue, we decided to evaluate the incidence of individual risk factors in the studied population. We compared their levels in patients with AMI and the control group (see Table 1). Ferritin was found as the most interesting risk factor. Serum ferritin concentration is an indicator of the amount of iron stored in the body. The correlation of higher iron concentration with the increased occurrence of AMI is in good accordance with the fact that metal ions may mediate radical perpetuation. Moreover, the protective iron-binding mechanisms may be diminished, or even lost, in partially damaged ischemic tissues due to decompartmentalization or disorganization (Healing *et al.* 1990). In fact, increased dietary iron concentration was found to cause oxidative changes in the plasma, erythrocytes and the liver (van Jaarsveld and Schulenburg 1997). The iron-supplemented diet can thus increase the degree of oxidative injury, while simultaneous antioxidant supplementation may prevent much of this negative effect (van Jaarsveld *et al.* 1994). For these reasons, our further work is focused on the estimation of the levels of individual non-enzyme antioxidants (vitamin

C and E, β-carotene) and evaluation of the impact of dietary supplementation with these substances. Fine balance of oxidant/antioxidant processes is necessary for maintenance and/or reestablishment of homeostasis. A correlation of insufficiency or loss of the activity of individual components of the antioxidant defense system with clinical manifestation of reperfusion injury will be the aim of our further studies.

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Reprint requests

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