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Antioxidant Vitamin Levels and Glutathione Peroxidase Activity During Ischemia/Reperfusion in Myocardial Infarction

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Summary

The consequences of increased oxidative stress, measured as the level of malondialdehyde (MDA) during ischemia/reperfusion, were studied in 48 patients in the acute phase of myocardial infarction (AMI) and a control group (21 blood donors). The serum levels of α-tocopherol and β-carotene were followed. Immediately after the treatment onset the level of α-tocopherol started to decrease, reaching a plateau after 24 h. The consumption of β-carotene was delayed by 90 min. Steady decline was detected during the whole time interval studied (48 h). Glutathione peroxidase (GPx) activity, as a representative of antioxidant enzymes, was estimated in whole blood. The influx of oxygenated blood was accompanied by a stimulation of GPx activity, which reached its maximum at the time of completed reperfusion. When comparing the AMI patients with the control group, the levels of MDA were found significantly increased, which indicates that oxidative stress is already increased during ischemia. Lower antioxidant levels found in the patients might either already be the result of vitamin consumption during ischemia or be a manifestation of their susceptibility to AMI. Monitored consumption of α-tocopherol and β-carotene during reperfusion indicated that in the case of patients, whose level of antioxidant vitamins is below the threshold limit, a further substantial decrease of antioxidant vitamins during reperfusion could enhance the oxidative damage of the myocardium.

Key words

Myocardial infarction • Oxidative stress • Malondialdehyde • Glutathione peroxidase • Alpha tocopherol • Beta carotene

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Introduction

The development of techniques which restore the flow of oxygenated blood to ischemic myocardial tissue has led to major advances in the treatment of AMI. It is now well established that early reperfusion by means of coronary bypass, transluminal angioplasty or thrombolytic therapy is essential for the survival of the ischemic myocardium. However, the restoration of blood flow may by itself increase the apparent severity of tissue injury. Several studies have shown that the generation of large amounts of reactive oxygen species (ROS) can occur upon post-ischemic reflow thus inducing a specific form of myocardial damage which is superimposed on ischemic injury (Davies *et al.* 1990, Young *et al.* 1993, Grech *et al.* 1993, Ambrosio and Tritto 1998).

The harmful reactive oxygen species are, to a smaller extent, also produced during normal cell metabolism. However, under certain pathological conditions their massive generation takes place. The organism possesses two major classes of cellular protection against ROS, which comprise a synergistic, multilevel defense system (Ferns *et al.* 1993). The enzymatic part is represented by free radical scavenger enzymes, namely superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Lin *et al.* 1997). The non-enzymatic part includes a large number of natural or synthetic antioxidant compounds (e.g. vitamins C and E, β-carotene, flavonoids) which have the ability to inhibit the oxidative damage by scavenging the highly destructive free radical species (Hess and Kukreja 1998).

As the direct measurement of liberated free radical species is limited by their instability, the level of malondialdehyde (MDA), the stable product of oxidative degradation of polyunsaturated fatty acids, has been widely adopted as a measure of free radical formation. In our previous study (Mužáková *et al.* 2000), an increase of the serum MDA in reperfused patients with AMI was found shortly after successful thrombolytical treatment. The increase was followed by rapid transient stimulation of plasma GPx activity. The specificity of the GPx response was confirmed by comparison with non-reperfused patients with AMI. In the present paper, these results were confirmed in a larger number of patients and supplemented with the data on the level of chain breaking antioxidants: α-tocopherol and β-carotene.

Alpha-tocopherol is the major lipid soluble antioxidant which is present in the blood, mainly in the VLDL and LDL fractions, where it prevents free radicals to oxidize these lipoproteins. When incorporated into the membranes, α-tocopherol protects myocardial phospholipids (Gutteridge 1995). Beta-carotene, another important lipid soluble membrane-bound antioxidant, is able to quench singlet oxygen interrupting the generation of ROS at a very early stage (Nagel *et al.* 1997).

The correlation of dynamics of ROS formation with the time course of glutathione peroxidase activity as well as of the antioxidant vitamin levels was followed in order to understand better of the consequences of reperfusion after acute myocardial infarction.

Material and Methods

Forty-eight patients (36 men, 12 women) in acute phase of myocardial infarction and 21 control subjects (17 men, 4 women) were studied (Table 1). The patients fulfilled the following criteria:
a) the first myocardial infarction (chest pain of more than 20 min duration, electrocardiographic changes consistent with AMI: pathological Q or at least 2 mm elevation of ST segment in two precordial or two inferior leads),
b) admission within 6 hours after the onset of AMI.

Table 1. Group characterization

	Acute myocardial Infarction	**Control group**
No. of patients	48	21
Age	60.7±10.6	55.5±4.1
Male/female	36/12	17/4
Body mass index [kg.m^{-2}]	27.5±4.0	22.6±4.9
Positive family history [%]	27.1	33.3
Total cholesterol [mmol.l^{-1}]	6.1±1.0	5.9±1.1
Index athero (according to Klimov)	4.2±1.6	3.9±1.3
Diabetes mellitus [%]	16.7	4.8
Hypertension [%]	56.2***	4.8
Cigarette smoking [%]	50.0	38.0
Ferritin [μg.l^{-1}]	144.1±111.8***	54.1±42.2
Fibrinogen [g.l^{-1}]	2.8±0.9***	1.5±0.2

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

Forty-three patients were treated by thrombolytic therapy (streptokinase: Streptase – Behringwerke Co., Germany,

1.5 MIU/h), 5 patients could not be treated in this way due to medical contraindications (enhanced risk of bleeding). Further treatment was prescribed as required. The control group included 21 blood donors of a similar age and of either sex, with no history of cardiovascular disease. A written informed consent was obtained from all the participants before starting the protocol and the study was approved by Hospital Committee on Human Research.

Peripheral venous blood samples were obtained from each patient immediately before the beginning of therapy and after 1.5, 3, 6, 12, 24 and 48 h. Samples were drawn into plastic tubes with heparin (Vacuette no. 456083, Greiner Labortechnik Co., Austria) for GPx estimation, and into plastic tubes coated with aluminium foil (to keep the samples in the dark) containing gel (Vacuette no. 455071, Greiner Labortechnik Co., Austria) for determination of MDA and both vitamins. Freshly frozen serum samples for MDA analysis were stored at –70 °C. MDA was estimated according to Hendrix and Assman (1990) by thiobarbituric acid (TBA) test. To achieve a high specificity, the absorbance of 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).

The activity of GPx was measured in the whole blood with the RANSEL set (Randox Co., Great Britain) using photometer Vitatron ISP (Vital Scientific, The Netherlands).

Vitamins were analyzed with HPLC (Ecom, CR) in a hexan extract evaporated under nitrogen atmosphere. Samples for α-tocopherol estimation were dissolved in methanol and separated on a Separon CGC SGX C_{18} column (150 mm, 3 mm i.d., 5 μm particle size, Ecom, CR) at a flow rate 0.5 ml/min with an isocratic gradient of acetonitrile-hexane-methanol (40:15:45, v/v/v). To determine the recovery internal standard tocopherol acetate was added. The UV absorbance was monitored on a UV-VIS detector (Ecom, CR), absorption at 290 nm being used for α-tocopherol quantification.

Beta-carotene was estimated in samples dissolved in a mixture of acetonitrile-hexane-methanol (40:15:45, v/v/v). This mixture was also used as a mobile phase. HPLC was performed using the same column as mentioned above. Detection was performed at 450 nm.

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

Reperfusion and the extent of myocardial injury were evaluated indirectly by measuring the time course of changes in CK and CK-MB isoenzyme activity.

The data are presented as mean values of the difference from the starting level (in %) ± S.E.M. Statistical significance of the differences during the time course was evaluated by 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.

Table 2 The levels of MDA, α-tocopherol, β-carotene and GPx in patients with AMI and in the control group

	Acute myocardial infarction	**Control group**
MDA [μmol. l^{-1}]	1.33±0.66***	0.79±0.25
α-tocopherol [μmol. l^{-1}]	20.70±7.23*	23.61±3.09
α-tocopherol/cholesterol [μmol. l^{-1}/ mmol.l^{-1}]	3.40±1.09*	3.97±0.95
β-carotene [μg. l^{-1}]	65.09±45.56**	98.55±0.10
GPx [U.gHb^{-1}]	38.55±13.41	41.86±10.30

*Data are expressed as means ± S.D., significantly different from the controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

Results

Reperfusion was successful in 34 patients, i.e. in 71 % of cases, in which maximum of CK and CK-MB activity was reached within 12 h after the administration of thrombolytic therapy (according to Zabel *et al.* 1993). In these patients the necrosis of myocardium was confirmed by a significant increase in CK, the peak of

which was 42.9±32.3 μkat.l^{-1}. Non-reperfused patients exhibited their CK peak later than 12 h after the onset of therapy (33.8±25.2 μkat.l^{-1}). Comparison of the patients with AMI and the control group is given in Table 1. No significant differences between the groups were found either in total cholesterol or in the ratio of cholesterol-HDL/HDL (index athero) which are considered as classical risk factors. High correlation of AMI incidence was found in case of hypertension, fibrinogen and ferritin levels.

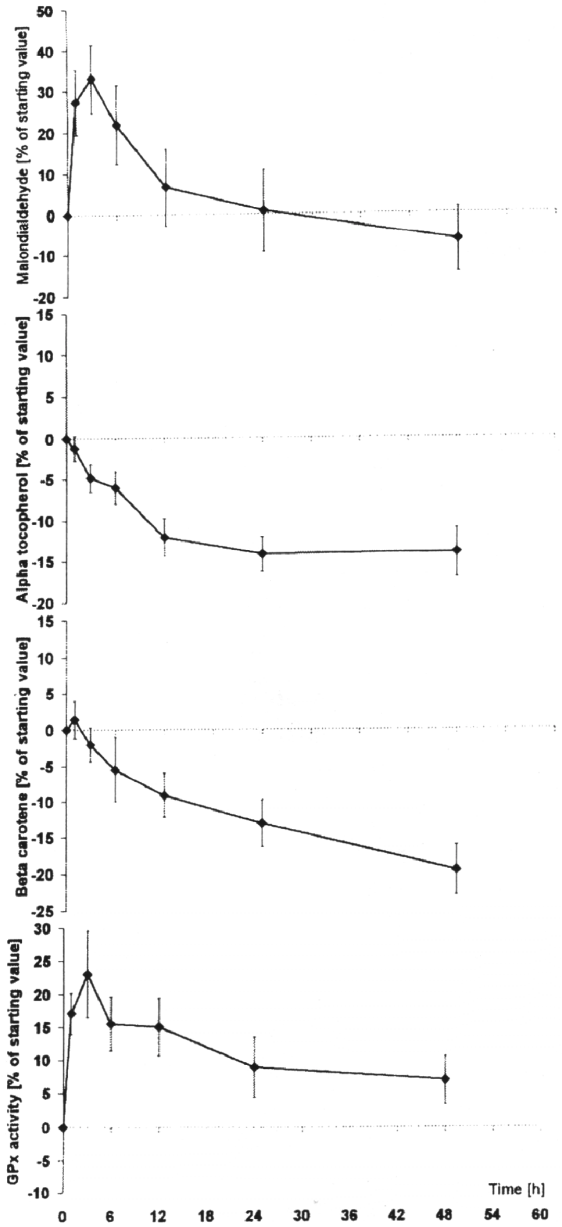

Fig. 1. *Malondialdehyde, α-tocopherol and β-carotene serum levels and glutathione peroxidase activity in reperfused patients with acute myocardial infarction. (Time 0 is time before the onset of therapy administration.) Data are means ± S.E.M.*

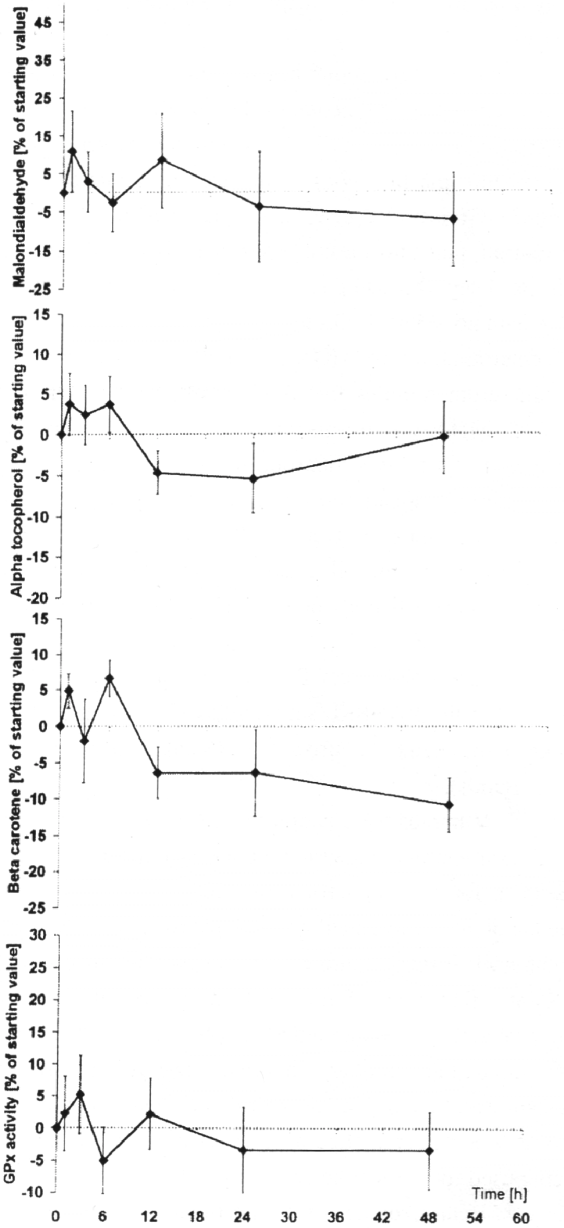

Fig. 2. *Malondialdehyde, α-tocopherol and β-carotene serum levels and glutathione peroxidase activity in non-reperfused patients with acute myocardial infarction. (Time 0 is time before the onset of therapy administration.) Data are means ± S.E.M.*

The starting values of MDA, α-tocopherol, β-carotene and GPx in AMI patients are compared with the control group (Table 2). The relatively high starting level of MDA, an indirect marker of lipid peroxidation and free radical activity, indicates that the extent of lipid peroxidation in AMI patients is already increased during the ischemic phase. Lower levels of antioxidant vitamins, especially of β-carotene, were simultaneously found in the patients. The activity of GPx was slightly lower in AMI patients in comparison with the control group (38.55±13.41 $U.gHb^{-1}$ vs. 41.86±10.30 $U.gHb^{-1}$). However, the detected difference was not statistically significant.

There was a significant rise in serum MDA in reperfused patients (n=34) from 1.30±0.72 μM before treatment to 1.61±0.98 μM after 3 h ($p<0.05$), when the maximum of oxidative stress was detected (Fig. 1). Later on a gradual decrease of MDA concentration was found. The baseline level was reached after about 24 h.

With the onset of reperfusion, a gradual decrease of α-tocopherol was detected in reperfused patients (from 20.52±6.91 μM at zero time to 17.53±6.19 μM after 24 h, $p<0.05$). This finding indicates that the consumption of this antioxidant vitamin during free radical elimination is increased in the case of successful reperfusion.

The level of β-carotene decreased from the initial value 66.12±49.86 $\mu g.\ l^{-1}$ to 52.37±43.77 $\mu g.\ l^{-1}$ after 48 h ($p<0.05$). The initiation of β-carotene consumption was delayed by approximately 1.5 h in comparison with vitamin E. While the α-tocopherol concentration reached a plateau after 24 h, the amount of β-carotene continued to decrease during the whole time period monitored.

The activity of the whole blood glutathione peroxidase (GPx) followed the same time course as MDA evolution (zero time: 38.31±13.88 $U.gHb^{-1}$, maximum at 3 h: 45.29±16.24 $U.gHb^{-1}$, $p<0.05$). This finding indicates that this antioxidant enzyme activity is rapidly enhanced by free radical formation.

The response of individual components of the antioxidant defense system in reperfused patients was confirmed by comparing the situation in non-reperfused patients with occlusion of a coronary artery (Fig. 2). In non-reperfused patients with prolonged hypoxia, MDA concentrations did not exhibit significant differences during the tested time interval (zero time: 1.43±0.48 μM, 3 h: 1.40±0.41 μM, $p>0.05$). The baseline levels of α-tocopherol and β-carotene differed substantially between individual patients, however, only a mild decrease, which did not reach statistical significance, was detected during the time course studied (α-tocopherol: zero time: 21.13±7.92 μM, 24 h: 19.78±7.35 μM, $p>0.05$; β-carotene: zero time: 62.62±33.03 $\mu g.\ l^{-1}$, 48 h: 54.12±26.79 $\mu g.\ l^{-1}$, $p>0.05$). The activity of GPx oscillated around the starting level and did not exhibit any unequivocal trend (zero time: 39.43±10.79 $U.gHb^{-1}$, 3 h: 40.71±13.17 $U.gHb^{-1}$, $p>0.05$).

Discussion

The reintroduction of molecular oxygen into ischemic tissue upon reperfusion has been proved to lead to excessive formation of ROS, which may overwhelm the tissue antioxidant defense capacity and damage myocardial cells. Ample evidence exists suggesting that free radicals are already produced to a limited extent during myocardial ischemia with a marked increase during the phase of reperfusion (Ferrari *et al.* 1985). During myocardial ischemia suppressed levels of the natural defense system were also detected (Chandra *et al.* 1994). Similar results have also been reported by Dhalla *et al.* (1999). These data are in concordance with our results summarized in Table 2. The basal level of MDA in patients with AMI was higher than that in the control group. In accordance with Singh *et al.* (1994) we found lower serum concentrations of both α-tocopherol and β-carotene in our patients. The question remains whether this might have contributed to the manifestation of myocardial infarction or whether it was a consequence of ischemia.

GPx was found to play a crucial role in myocardial protection from ischemic reperfusion injury (Molina and Garcia 1997, Yoshida *et al.* 1997). Decreased GPx activity in human cardiomyocytes was found to be consistent with increased susceptibility to oxidant injury (Li *et al.* 1994), while overexpression of the gene for GPx made the mouse heart more resistant to myocardial infarction-reperfusion injury (Yoshida *et al.* 1996). Our data on the stimulation of GPx activity in the early phase of reperfusion are in accordance with the findings of Chandrasekar *et al.* (1997). These authors reported that the increase of GPx activity might be mediated by rapid enhancement of antioxidant enzyme gene expression by the reperfusion. Our results on the stimulatory effect of ROS on the GPx activity are in good correlation with the results of McDonough (1999) and Atalay and Sen (1999), who pointed out the interesting practical impact of this phenomenon. Repeated exposure

to the mildly elevated levels of ROS caused by augmented demands for ATP under conditions of an increased work load increased the antioxidant production, which might result in myocardium adaptation and consequently mitigate the damage caused by ischemia reperfusion injury. However, prolonged exposure to oxidative stress was found to result in depletion of the defense system which might decrease GPx activity (Li *et al.* 1998). It is interesting that, according to our findings, the basal level of GPx was lower in patients during ischemia than in the control group; however, this difference was not statistically significant. Similar results were achieved by Bor *et al.* (1999).

It seems that both parts of the antioxidant defense system, the enzymatic and non-enzymatic, are interrelated. For example, α-tocopherol was reported to stabilize GSH-Px1 mRNA posttranscriptionally (Li *et al.* 1996).

Some epidemiological studies have shown an association between high dietary intake or high serum concentrations of α-tocopherol or β-carotene and lower rates of ischemic heart disease (e.g. Kardinaal *et al.* 1993, Stampfer and Rimm 1995, Kushi *et al.* 1996). However, large randomized controlled trials, such as the CHAOS (Stephens *et al.* 1996) or ATBC study (Rapola *et al.* 1997), did not prove an unambiguously positive effect of α-tocopherol and β-carotene supplementation on the incidence of coronary events. In spite of these rather contradictory results, some experimental studies confirmed the beneficial effect of vitamin E. Tripathi and Hegde (1997) reported that α-tocopherol pretreatment significantly reduced the myocardial infarct size and the relative proportion of myocardial necrosis in dogs. Vitamin E supplementation was found to inhibit ROS formation in rat mitochondria (Scholz *et al.* 1997) and/or to improve hemodynamic function in rats with AMI (Palace *et al.* 1999).

Especially in the case of low endogenous α-tocopherol levels its administration was reported to exert a strong protection against lipid peroxidation (Rojas *et al.* 1996). The need for vitamin E dietary supplementation was found important for improving the antioxidant capacity of the mammalian heart, which has constitutively a lower level of this capacity than other organs. When 120 patients with AMI were analyzed for vitamin E and β-carotene levels, Carrasquedo *et al.* (1999) found that a higher concentration of α-tocopherol, but not of β-carotene, was associated with lower creatine phosphokinase release and AMI extension. Spencer *et al.* (1999) concluded that vitamin E levels below the threshold limit constitute a risk factor for coronary artery disease, while supplementation at the levels above this limit did not provide any additional protection. This conclusion is in accordance with our finding that α-tocopherol levels are gradually decreasing during reperfusion. A significant drop in plasma vitamin E concentration upon reperfusion was also found by Levy *et al.* (1998) and Dusinovic *et al.* (1998). Increased ROS production together with decreased antioxidant levels (vitamin E, retinol) after reperfusion were already reported by Young *et al.* (1993). Together with the consumption of vitamin E, we found decreased β-carotene levels. This is consistent with the results of Street *et al.* (1994) who found positive correlation between a low β-carotene level and an increased risk of AMI among smokers. High dietary β-carotene intake was found to protect against cardiovascular diseases (Klipstein-Grobusch *et al.* 1999).

It may be concluded from the above data that antioxidant vitamin levels below the threshold limit may be a risk factor of AMI. The analysis of vitamin levels and the supplementation based on their actual levels of individual patients may have a considerable protective impact. The problems of antioxidant vitamin deficiency seems to be a very important factor especially in the Czech population due to the low dietary intake of vegetables and fruits containing antioxidant vitamins. In an extensive study, Bobak *et al.* (1999) compared the wide range of risk factors in random samples of Czech and Bavarian men, which differed substantially in the occurrence of coronary heart disease. The main differences identified were low levels of carotenoids and high concentrations of homocysteine in the Czech population. In spite of the fact that the Czech dietary habits have been changing in a positive way since 1989, the low intake of antioxidant vitamins still seems to be an indispensable factor in coronary heart disease.

Our results on vitamin E and β-carotene levels during reperfusion in AMI patients should be supplemented with the data concerning another antioxidant vitamin – vitamin C. Their correlation with the clinical course of AMI will be the subject of the following study.

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