Original Article

Gelatinases A and B and Antioxidant Enzyme Activity in the Early Phase of Acute Myocardial Infarction

(acute myocardial infarction / biomarkers / gelatinase A / gelatinase B / oxidative stress)

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Abstract. Oxidative stress plays important roles in the pathophysiology of acute myocardial infarction (AMI). The aim of this study was to investigate the correlation of the oxidative stress status and matrix metalloproteinase activity in AMI patients in comparison to controls. This study included 136 subjects: 68 patients with AMI (42 males/26 females; mean age 58.5 ± 10.5 years) and 68 controls (37 males/29 females; mean age 60.2 ± 12.4 years). Gelatinases A and B were assayed using gelatin zymography, enzyme activities were obtained spectrophotometrically. Gelatinase A and B activities were increased in the AMI patients' group compared to the control. Activities of serum superoxide dismutase (SOD) and xanthine oxidase (XO) were significantly higher in AMI patients (106.53 ± 23.45 U/l, P < 0.001 and 158.18 ± 29.59 U/l, P < 0.001) than in the control group (55.99 ± 10.79 U/l and 79.81 ± 7.93 U/l). The activity of catalase (CAT) in the sera of AMI patients was lower (271.31 ± 7.53 U/l, P < 0.005) than in the control group (305.94 ± 97.28 U/l). Plasma glutathione peroxidase (GPx) and glutathione reductase (GR) in AMI patients were significantly higher (582.47 ± 184.81 U/l, P < 0.001 and 59.64 ± 21.88 U/l, P < 0.001) than in the control group (275.32 ± 104.69 U/l and 47.71 ± 20.05 U/l). The present findings demonstrate activation of gelatinases A and B and oxidative stress markers in the early stage of AMI. Gelatinases, detected at high levels in AMI patients only, indicate their noticeable predisposition for becoming additional biomarkers of the early phase of AMI.

Received April 14, 2016 Accepted October 14, 2016

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Abbreviations: AMI – acute myocardial infarction, CAD – coronary artery disease, CAT – catalase, GPx – glutathione peroxidase, G6PD – glucose-6-phosphate dehydrogenase, GR – glutathione reductase, GST – glutathione-S-transferase, LAD – left anterior descending artery, MMPs – matrix metalloproteinases, NBT – nitroblue tetrazolium, NO – nitric oxide, NOx – nitrates and nitrites, ROS – reactive oxygen species, SD – standard deviation, SOD – superoxide dismutase, STEMI – ST elevation myocardial infarction, XD – xanthine dehydrogenase, XO – xanthine oxidase.

Introduction

Coronary artery disease (CAD) followed by acute myocardial infarction (AMI) still represents a significant cause of death in high-income countries and is the second most common cause of death in medium- and low-income countries. Within the complex aetiology of CAD are thrombotic events following atherosclerotic plaque rupture with subsequent occlusion of the coronary artery, leading to AMI (Chan and Ng, 2010). Increased values of specific circulating biomarkers of AMI, such as creatine kinase-MB and cardiac troponins I and T, confirm myocardial necrosis (Thygesen et al., 2012). Many reports suggest that reactive oxygen species (ROS) initiate and potentiate atherosclerosis, leading to CAD.

Redox enzymes (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx, glutathione reductase – GR, glutathione-S-transferase – GST and glucose-6-phosphate dehydrogenase – G6PD) can neutralize the damaging effects of endogenous ROS on biomacromolecules (Halliwell, 2012). Oxidative stress also induces changes in Na^+, K^+ and Ca^{2+} ion channels located in sarcoplasmic reticulum. During ischaemia, increased intracellular Ca^{2+} concentrations stimulate conversion of xanthine dehydrogenase (XD) into xanthine oxidase (XO) with production of large amounts of superoxide radicals (Tripathi et al., 2009). Another mecha-

nism that may lead to CAD is a defect in synthesis or activity of nitric oxide (NO). When released from dying cells into extracellular space, it is rapidly auto-oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). The total amount of NO_2^- and NO_3^- termed NOx may be an indicator of endogenous formation of NO (Hare and Stamler, 2005).

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent metalloendopeptidases that cleave or degrade numerous components of the extracellular matrix, as well as a large variety of other extracellular proteins, during normal and disease conditions (Ra and Parks, 2007). Of special interest for the vasculature and vascular disease are gelatinase A (MMP-2, 72 kDa) and gelatinase B (MMP-9, 92 kDa), which readily digest collagen type IV, fibronectin, elastin and other molecules within the vascular wall (Liu et al., 2006). Both gelatinases are secreted as inactive pro-forms (proMMPs) in physiological conditions and they are activated within the extracellular matrix in plaques through mechanisms that are not fully understood. Following inactivation, their activity is primarily regulated by tissue inhibitors of MMPs (TIMPs) that bind to the enzyme active site and prevent substrate availability (Kurzepa et al., 2014). The activity of MMPs has been shown to increase with progression of various cardiovascular pathological conditions. Several studies have considered a link between redox imbalance and MMP activation during the onset of AMI, but the data obtained are not consistent (Kameda et al., 2003; Yabluchanskiy et al., 2013). To address these shortcomings, the objective of the present study was to observe the possible association between the activity of gelatinases A and B and the oxidative stress status in AMI patients in comparison to healthy controls.

Material and Methods

Study group

Patients were recruited from the Emergency Centre, Clinic of Cardiology, Clinical Centre of Serbia. The study was conducted at the Department of Chemistry in Medicine, Faculty of Medicine, University of Belgrade, from November 2009 to April 2012 and the Department of Biochemistry, Faculty of Medicine, University of Nis, Serbia, from March 2010 to November 2011.

Patients were selected consecutively. Two groups were formed as follows: the experimental group included 68 patients of both sexes (42 males, 26 females) with AMI within an age interval of 58.5 ± 10.5 years. AMI was established by clinical diagnosis, based on specific ECG changes, creatine-kinase MB and troponin T. All patients were diagnosed for ST elevation myocardial infarction (STEMI) of the left anterior descending artery (LAD). Patients with a previous medical history of cardiovascular, metabolic (diabetes mellitus), renal, lung, neoplastic, autoimmune and infectious diseases, as well as chronic drug users and smokers, were not included. Blood samples were drawn within 5 h from the moment of acute chest pain.

The control group consisted of 68 healthy, non-smoker individuals, who were individually matched by age (60.2 ± 12.4 years) and sex (37 males, 29 females) with participants in the experimental group. Serum and plasma were obtained from peripheral blood collected by cubital venepunction and stored at –20 °C until required. Aliquots of serum and plasma were then thawed by incubation at 25 °C for 15 min. Before the enzyme assays all samples were incubated at 37 °C for 15 min. Each assay was performed in triplicate. Activities of SOD, CAT, XO, NOx, gelatinases A and B were determined using the serum, while GPx and GR activities were measured in the plasma. Each sample from AMI patients and controls was evaluated for all defined parameters at the same time. The study protocol was approved by the Institutional Ethics Committee, and all subjects gave written informed consent.

Biochemical parameters

Troponin T was estimated by chemiluminescence immunoassay in a Siemens dimension RXL max analyser (Brookfield, CT), reference value < 0.3 ng/ml. Serum SOD activity was determined according to the method of Sun and Zigman (1978) by measuring the absorbance change during autooxidation of adrenalin into adrenochrome at 340 nm. Commercial SOD from human erythrocytes (Sigma-Aldrich Darmastadt, Germany) was used as standard for evaluation of the total SOD activity. Activities of SOD isoforms were obtained using reverse electrophoretic zymography following the method of Flohe and Otting (1984). The protein concentration in each sample was determined according to Bradford's method and equal amounts of protein were loaded into wells of a gel (Bradford, 1976). Isoforms appeared as two transparent bands (cytosolic Cu^{2+}/Zn^{2+} SOD and mitochondrial Mn^{2+} SOD) after staining the gels with nitroblue tetrazolium (NBT) and contrasting with TEMED.

The positive control contained equal amounts of human recombinant cytosolic and mitochondrial SOD isoenzymes (Sigma-Aldrich). As a negative control, SOD was mixed with a 5 mmol/l solution of EDTA (10 µl of SOD and 10 µl of EDTA). After electrophoretic zymography of SOD, gels were densitometrically analysed by ImageJ 1.42q software package. Relative activities of Cu^{2+}/Zn^{2+} SOD and Mn^{2+} SOD were expressed as a percentage of total activity (relative activity), which was taken as 100 % activity. Serum CAT activity was determined spectrophotometrically according to the method of Beers and Sizer (1952). The absorbance change during hydrogen peroxide breakdown by CAT was measured at 240 nm. Commercial CAT from human erythrocytes (Sigma-Aldrich) was used as standard for evaluation of the total CAT activity. Plasma GPx activity was determined spectrophotometrically according to the method of Wendel (1981). The absorbance change was measured during oxidation of NADPH at 340 nm. Commercial GPx from human erythrocytes (Sigma-Aldrich) was used as standard for evaluation of the total GPx activity. Plasma GR was determined spectrophoto-

metrically using the method of Carlberg and Mannervik (1985). The absorbance change during oxidation of NADPH was measured at 340 nm. Human recombinant GR (Sigma-Aldrich) was used as standard for estimation of the total GR activity. Serum XO activity was determined spectrophotometrically by measuring the absorbance change at 290 nm during uric acid oxidation to xanthine (Westerfeld et al., 1959). The commercial XO Kit (Sigma-Aldrich) contained standard XO enzyme for protocol calibration.

Gelatin zymography

SDS-PAGE reverse electrophoretic zymography was used to determine gelatinase A and B activities (La Rocca et al., 2004). Protein concentration was quantified according to the Bradford method and equal amounts of protein were loaded on the gel for each sample. After 72 h of renaturation, gels were stained with Coomassie brilliant blue G-250 (CBB G-250) dye and regions of gelatinase A and B activity appeared as transparent areas on a blue background. The negative control consisted of sera incubated with 5 mmol/l EDTA. Human recombinant gelatinases A and B (R&D Systems, Minneapolis, MN) were employed as standard. The negative control contained a mixture of standard human recombinant gelatinases A and B, and 5 mmol/l EDTA. After electrophoretic zymography the gels were analysed densitometrically using an ImageJ 1.42q software package. Relative activities of gelatinases A and B were expressed as percentages of total activity, which was taken as 100 %. The relative activities of the gelatinases were compared using Student's *t*-test.

Determination of nitrites and nitrates

Serum levels of cumulative production of nitrates and nitrites were determined spectrophotometrically, using a modified cadmium-reduction method based on the Griess reaction (Navarro-Gonzalez et al., 1998). The commercial NOx Kit (Sigma-Aldrich) contained a standard solution (100 mmol/l) of sodium nitrate and sodium nitrite for protocol calibration. The concentration of total nitrates and nitrites was expressed as μmol per litre of serum.

Statistical analysis

The data are expressed as the means ± standard deviation (SD) obtained from triplicates in one experiment. Data distribution was examined using the Kolmogorov-Smirnov test. The existence of statistically significant differences between the AMI and control groups was inferred by Student's *t*-test for SOD, CAT, GPx, GR, XO and NOx. Student's *t*-test was also used to evaluate differences between relative gelatinase A and B activities within the AMI group, as well for the relative activities of SOD isoforms in both groups. Pearson's and Spearman's correlation tests were used to examine associations between study variables. All statistical analyses were performed using an SPSS 20.0 software package. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

Serum activities of gelatinases A and B

Gelatinase A and B activities were recorded in the serum of each patient with AMI, but were not detected in any individual from the control group, providing striking 100 % sensitivity for both gelatinases during the early phase of AMI. A representative graph of average relative activities of gelatinases A and B and a representative zymogram are presented in Fig. 1.

Gelatinase A (72 kDa) was detected as a band of faster mobility, while gelatinase B (92 kDa) had slower electrophoretic mobility. The average relative activity of gelatinase A was 77.1 % and that of gelatinase B was 22.9 % in the AMI patients' group. The difference in activity between gelatinase A and gelatinase B was statistically highly significant in the group of AMI patients ($P < 0.001$). We tested the relative activities of gelatinases A and B and enzymes of oxidative stress for correlation but found none. Also, associations between the mean levels of creatine-kinase MB and troponin T (26.9 ± 9.4 ng/ml; 0.059 ± 0.021 ng/ml, respectively) and gelatinases A and B were examined in the AMI group. Gelatinase A activity was not correlated with the levels of creatine-kinase MB and troponin T ($r = 0.221$, $P > 0.05$; $r = 0.433$, $P > 0.05$, respectively), nor did gelati-

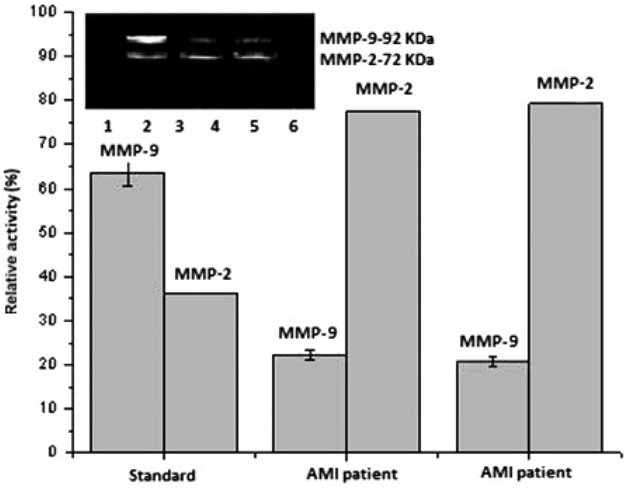

Fig. 1. Graph of standard gelatinase A and B activities and activities of gelatinases in the sera of AMI patients. The average relative activity of gelatinase A was 77.1 % and of gelatinase B 22.9 % in the group of AMI patients. Data were obtained after densitometric analysis of the isoforms separated by gelatin electrophoretic zymography.
Zymograms of gelatinases A and B in the sera from AMI patients and the control group. Lane 1: negative control, both standard MMPs with EDTA; lane 2: positive control, human recombinant gelatinases A and B; lanes 3 and 4: AMI patients; lane 5: control individual.

Table 1. Activities of serum SOD, CAT and XO, and plasma GPx and GR of patients with AMI and the control group

Parameter (U/L ± SD)	Control group (N = 68)	AMI patients (N = 68)	P value (AMI *vs* C)
SOD	55.99 ± 10.79	106.53 ± 23.45	P < 0.001
CAT	305.94 ± 97.28	271.31 ± 87.53	P < 0.005
GPx	275.32 ± 104.69	582.47 ± 184.81	P < 0.001
GR	47.71 ± 20.05	59.64 ± 21.88	P < 0.001
XO	79.81 ± 7.93	158.18 ± 29.59	P < 0.001

C – healthy control; significance level: P < 0.05 significant; P < 0.001 highly significant

nase B correlate with the levels of creatine-kinase MB or troponin T (r = 0.312, P > 0.05; r = 0.778, P > 0.05, respectively).

Antioxidant enzyme activity

Table 1 shows the activities of serum SOD, CAT and XO and plasma GPx and GR in patients with AMI and the control group.

The findings presented in Table 1 indicate that a redox imbalance is present in the early phase of AMI. Namely, the enzymatic profile demonstrates that redox imbalance occurs at different levels of ROS production in the circulation of AMI patients. In this patient group, the plasma activity of GPx was positively correlated with CAT activity (r = 0.771, P < 0.01). This finding points to an overall anti-peroxide response, resulting from ischaemic-reperfusion injury, followed by ROS production and increased SOD activity. Statistical analysis of the data presented in Table 1 is displayed in Table 2.

The Kolmogorov-Smirnov test showed that all examined enzymatic parameters were normally distributed. All parameters were evaluated in relation to gender, and no significant differences between the sexes were found (Table 2).

Distribution of SOD isoforms

Both Cu^{2+}/Zn^{2+} SOD and Mn^{2+} SOD isoforms were detected in AMI patients and the control group as evident in Fig. 2, which shows a representative zymogram of SOD isoform activities.

The relative activity of Cu^{2+}/Zn^{2+} SOD and of Mn^{2+} SOD in the AMI patients' group differed significantly from the values of the control group (P < 0.001). Mn^{2+} SOD was the dominant SOD isoform in the sera of both groups. The Mn^{2+} SOD isoform accounted for 82.5 % of the relative activity and the Cu^{2+}/Zn^{2+} SOD isoform for 17.5 % in the AMI patients' group. In the control group, the relative activity of the Mn^{2+} SOD isoform was 81.8 % and of Cu^{2+}/Zn^{2+} SOD isoform 18.2 %. There was no statistically significant difference (P > 0.05) between the distribution of relative activities of SOD isoforms between the AMI patients' group and the control group (Table 2).

Total serum nitrate and nitrite concentrations

As the product of NO oxidation, cumulative production of nitrates and nitrites (NOx) was slightly greater in the AMI patients' group (131.1 ± 26.3 μmol/l) than in

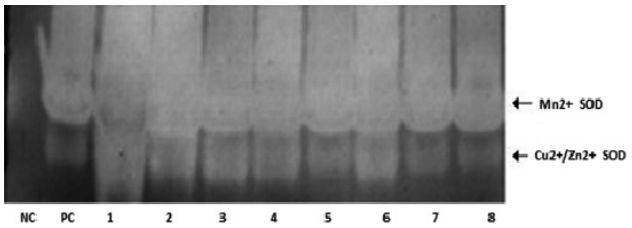

Fig. 2. Representative zymogram of SOD isoforms in the sera of AMI patients and the control group. NC – negative control, human recombinant SOD isoforms with EDTA; PC – positive control, human recombinant SOD isoforms; lanes 1 to 6 – SOD isoforms in AMI patients; lanes 7-8 – control group individuals. The average relative activity of SOD isoforms in the sera of AMI patients was: 82.5 % for the Mn^{2+} SOD isoform and 17.5 % for the Cu^{2+}/Zn^{2+} SOD isoform. Data were obtained after densitometric analysis of SOD isoforms separated by reverse electrophoretic zymography.

Table 2. Statistical analysis of the data presented in Table 1

SOD	CAT	GPx	GR	XO	NOx	MMP-2 and -9
P < 0.001* Cu^{2+}/Zn^{2+} SOD *vs.* Mn^{2+} SOD P < 0.001* (AMI and control) SOD_{AMI} *vs.* $SOD_{control}$ P > 0.05*	P < 0.005*	P < 0.001*	P < 0.001*	P < 0.001*	P > 0.05*	P < 0.001* AMI group
P = 0.782**	P = 0.566**	P = 0.477**	P = 0.891**	P = 0.822**	P = 0.921**	P = 0.523** P = 0.322**
SOD *vs.* NOx r = –0.678, P<0.05*** GPx vs. CAT r = 0.771, P<0.01***						

*Student's t -test; **Kolmogorov-Smirnov test, distribution; ***Pearson's correlation test

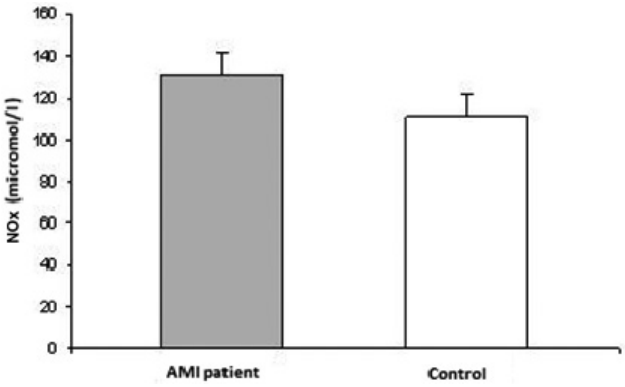

Fig. 3. Total amount of NOx in the sera of AMI patients and the control group. Data were obtained spectrophotometrically using a modified Griess reaction, as described in Material and Methods, and are expressed as μmol (nitrites and nitrates)/l. The results are means ± S.D. (or S.E.M.) (N = 68 for AMI and N = 68 for the control group). Statistical analysis was performed using the SPSS 12.0 software package. Differences are considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

healthy persons (111.2 ± 23.8 μmol/l), but the increase was not statistically significant ($P > 0.05$; Fig. 3).

When the results for enzyme activities are taken into consideration, this small effect is probably a consequence of the compensatory increased activity of SOD and GPx, which act against formation of the oxidation products of NO. The negative correlation ($r = –0.678$, $P < 0.05$) between SOD activity and NOx concentration only among AMI patients supports this finding (Table 2).

Discussion

This study reports activation of gelatinases A and B in the sera of patients with AMI involving oxidative stress. The role of various matrix metalloproteinases, including gelatinases A and B, in various cardiovascular diseases is well documented (Agewall, 2006). It has been shown that gelatinase A has an important role in remodelling and regulation of the extracellular matrix and that its main source is represented by ruptured atherosclerotic plaque. Another important condition for activation and release into the circulation is oxidative stress in the myocardium during AMI-induced ischaemia (Phatharajaree et al., 2007). In their prospective study on non-ST segment elevation acute coronary syndrome, Bittner et al. (2010) showed that enhanced synthesis of gelatinase B is associated with oxidative stress in patients with acute coronary syndrome. MMP activity increased significantly within hours of infarction, accompanied by local activation of cytokines and infiltration of inflammatory cells into the circulation (Nian et al., 2004). The mechanisms of activation of proMMPs into MMPs still remain unclear and controversial. Since we detected gelatinase A and B activity only in the sera of AMI patients, our data strengthen the notion that AMI is accompanied by expression of enzymes that enact tissue remodelling with subsequent oxidative and nitrosative stress. The increase of serum SOD activity in patients with AMI could participate in the defence against free radicals leading to reduction of myocardial damage caused by oxidative stress. Wang et al. (1998b) demonstrated lower activity of plasma SOD in patients with a history of AMI. However, in their clinical study, Horiuchi et al. (2004) showed that vascular SOD expression was significantly higher on the first day after the onset of AMI.

These apparently contradictory results could be due to different times of blood sampling and, together with our findings for samples taken within 5 h after chest pain, suggest a transient rise in SOD, followed by lowered activity. On the contrary, using the animal model, Tavares et al. (2012) showed that SOD was preserved in heart homogenates at 48 h post AMI. The dominant SOD isoform in the sera of both AMI patients and control subjects is Mn^{2+} SOD. As Perrelli et al. (2011) reported, ischaemic heart injury induces mitochondrial permeability transition pore (mPTP) opening, permitting communication between the cytoplasm and the mitochondrial matrix. Usui et al. (1991) found that serum Mn^{2+} SOD activity increased gradually after the first hospital day. Moreover, using a transgenic mouse model, it has been demonstrated that higher expression of Cu^{2+}/Zn^{2+} SOD prevents post-ischaemic injury following reperfusion (Wang et al., 1998a).

In our study group a slight elevation in the serum Mn^{2+} SOD activity was found in the group of AMI patients, but the lack of statistical significance was probably due to early blood sampling. The small decline in CAT activity among AMI patients was also not statistically significant and may have been unnecessary due to the activation of GPx. GPx has higher affinity toward peroxide and thus would have scavenged most radicals produced. Alternatively, CAT activity may have decreased due to exhaustion and/or oxidative damage caused by increased peroxidation, as a consequence of raised concentration of H_2O_2 with which it rapidly reacts to form water and oxygen. Cabigas et al. (2014) found significant enhancement of catalase activity following infarction with a subsequent decline in the H_2O_2 level, leading to improved acute function. Possible cooperative action of GPx and CAT in patients with AMI is suggested by the close positive correlation between their activities. After AMI, CAT levels, although stable initially, were found to decrease over time, whereas GPx remained unchanged (Pendergrass et al., 2011). Because most of the peroxidase activity in myocytes originates from CAT, this significant change can drastically alter the redox balance in the myocardium and makes a strong case for restoration of the myocardial CAT level as an attractive therapeutic option. Enhanced GR activity among AMI patients could result from the greater need for reduction of oxidized glutathione formed by GPx.

Discrepant results for the antioxidative enzyme activity in the sera of early-phase AMI patients have been obtained previously (Elahi et al., 2009). The highly sig-

nificant increase in XO activity in the sera of AMI patients supports the notion of free radical-mediated damage and indicates pathological activation of the main source of oxygen free radicals in the circulation. Bergsland et al. (1987) demonstrated that XO inhibition with allopurinol prevented reperfusion injury in rat hearts, underlining the role that XO has in cardiac redox disequilibrium and progressive myocardial damage during AMI. We recorded a slightly higher NOx concentration in AMI patients than in healthy persons, but the increase was not significant. This could be a consequence of early blood sampling and/or the compensatory effect of increased SOD and GPx activity. The significant negative correlation between NOx concentration and SOD activity supports this causal relationship. Not only is an imbalance between NO and ROS a consequence of AMI, but it is also considered to be a major contributor to endothelial dysfunction in atherosclerosis and cardiac disease (Lundberg and Weitzberg, 2009).

Our findings strengthen the hypothesis that redox imbalance is an activator of pro-MMPs in atherosclerotic plaques. Potential inhibition of increased gelatinase A and B activity with TIMPs could possibly act as a therapeutically protective factor by reducing the level of gelatinolytic activation, and therefore the extent of myocardial damage caused by proteolytic cleavage of the cardiac extracellular matrix. We propose further investigation of gelatinases A and B as diagnostic and prognostic markers of AMI, especially when precise quantification of their activities and correlation with clinical parameters are concerned.

Acknowledgment

We are very grateful to Mira Susa, PhD for helping us with manuscript preparation.

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