TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2015) 45: 148-152

doi:10.3906/sag-1308-25

Stress-responsive factor regulation in patients suffering from type 2 diabetes and myocardial infarction

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Received: 06.08.2013 • **Accepted:** 19.03.2014 • **Published Online:** 12.01.2015 • **Printed:** 09.02.2015

Background/aim: Pro-free radical oxidative stresses, as well as regulatory factors, are believed to be the key players in the development of diabetes and heart-related disorders such as myocardial infarction. The aim of the present study was to highlight the role of oxidative stress-responsive factors (reactive oxygen species [ROS], super oxide dismutase [SOD], and calpain-1) in type 2 diabetes and myocardial infarction.

Materials and methods: A total of 100 type 2 diabetes patients with myocardial infarction and 50 normal individuals were selected for this analysis. The levels of ROS and activities of SOD in the serum were determined. Serum calpain-1 expression was checked using western blotting.

Results: The serum level of ROS and the expression of calpain-1 were significantly higher while the activity of SOD was significantly lower in diabetic patients with myocardial infraction compared to normal individuals.

Conclusion: These findings suggest a possible link between decreased antioxidant (SOD) and increased ROS levels as well as calpain-1 expression, supporting the role of oxidative stress-regulatory factors in diabetes and myocardial infraction.

Key words: Diabetes mellitus, myocardial infraction, super oxide dismutase, reactive oxygen species, calpain-1

1. Introduction

The term 'myocardial infarction' (MI) reflects the death of cardiac myocytes. Myocardial cell death can be predicted by the appearance of proteins that are generated by the damaged myocytes (1). Mortality rates of diabetic patients due to acute myocardial infarction are significantly higher compared to nondiabetic patients, posing a serious threat to diabetics (2–4). Persistent hyperglycemia leads to the development of atherosclerosis. Within the endothelium, hyperglycemia prevents nitric oxide production and its bioavailability, leading to increased oxidative stress (5).

Reactive oxygen species (ROS), produced during normal aerobic metabolism, are involved in signal transduction and cognitive functions, but exceedingly increased ROS concentrations also have harmful effects (6). The potential sources of ROS production in the cardiovascular system are numerous, consisting of mitochondria, NADPH oxidase, xanthine oxidase, activated neutrophils, NO synthases, and cyclooxygenases (7), which lead to ischemia/reperfusion injury and an increase in inflammatory cytokines (8). The free radicals are neutralized by antioxidant action of both nonenzymatic as well as enzymatic antioxidants (9). Superoxide dismutase (SOD) works as the first line of defense against ROS. Recent studies have demonstrated that extracellular SOD expression is decreased in the failing heart, and this is associated with increased myocardial oxidative stress and endothelial dysfunction (10).

Previous studies have documented the independent role of ROS and calpain in myocardial cell deaths. Calpain-1 is a ubiquitous cytosolic Ca^{2+} activated neutral protease (11,12). The physiological role of calpain-1 in organs like the heart and its mechanism in diseased hearts are not yet known, but studies indicate that calpain is activated by Ca^{2+} overloads in diseased hearts, resulting in cardiac dysfunction (13). Calpain-1 is involved in apoptosis by cleaving Bax and promotes its proapoptotic activity (14–17). Calmodulin and calpastatin are the 2 inhibitors of calpain that reduce the expression and activity of calpain (18,19). These inhibitors get proteolyzed during ischemia, leaving calpain free to act on substrates. Calpain is also involved in the apoptotic machinery by the activation of caspase-12 and cytochrome c (20,21).

The present study delineates the expression of oxidative stress regulators including ROS, SOD, and calpain in diabetic MI. Regulation of their activities by ROS

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scavengers and calpain inhibitors may not only contribute in preventing cellular damage but may also contribute in regulating diabetes in its shift to MI at varying stages.

2. Materials and methods

2.1. Blood sample collection and serum extraction

All human sample collections were made according to ethical regulations of the respective institutes. Blood samples for biochemical analysis were collected in Vacutainer tubes and immediately stored at 4 °C. Serum was then separated as a supernatant by centrifugation at 4000 rpm for 5 min and stored at –20 °C until analysis.

2.2. ROS analysis of serum samples

The serum free radical level measurements were carried out as follows (22). Briefly, N,N-diethyl-pera-phenylenediamine (DEPPD) (R1) was dissolved in 0.1 M sodium acetate buffer (pH 4.8) to get a final concentration of 100 µg/mL and ferrous sulfate was dissolved in sodium acetate buffer to attain a final concentration of 4.37 µM (R2). R1 and R2 were mixed at a ratio of 1:25. This solution was added as starter in a cuvette (3 mL) followed by a sodium acetate buffer. Hydrogen peroxide (H_2O_2) was added as a positive control and serum samples were analyzed for ROS detection at 505 nm. Increase in absorbance was related to elevated ROS concentrations.

2.3. SOD analysis of serum samples

SOD analysis of samples was carried out with modifications as described previously (23). A reaction mixture was prepared by taking phosphate-buffered saline (NaCl, KH_2PO4, Na_2HPO4, and KCl), L-methionine, nitro blue tetrazolium (NBT), and Triton X-100 followed by the addition of the sample. After illumination with a fluorescent lamp, riboflavin was added to initiate the reaction. The sample mixture was delivered into cuvettes and a measurement was taken at 560 nm. A control sample without serum was also used in parallel.

2.4. Western blotting analysis

Expression of calpain-1 in patients and normal individuals was assessed by western blotting. Serum samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose-supported transfer membrane with pore size of 0.45 µm (Santa Cruz Biotechnology, USA) at 100 V for 2 h in a transfer buffer (25 mM Tris, 192 mM glycine, and 20% methyl alcohol, pH 8.3) according to the manufacturer's instructions. After being blocked with defatted milk, the membrane was incubated overnight with a primary antibody (Santa Cruz Biotechnology) using working dilutions of 1:1000 at 4 °C and then with goat antirabbit IgG-AP at 1:10,000 dilutions (Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was stained for 30 min in alkaline phosphatase (AP) color development solution containing 1 mL of 1X AP reaction buffer with 50 µL of NBT solution and 50 µL of 5-bromo-4-chloro-3-indolyl phosphate solution (Tiangen, China).

2.5. Statistical analysis

Statistical analysis was performed using SPSS 16. Multiple linear regression tests were applied to calculate the significant values. $P < 0.05$ was considered significant.

3. Results

3.1. Serum ROS level in diabetic MI patients

Patients suffering from type 2 diabetes and MI in combination were considered as study subjects. In the current study, 100 patients and 50 healthy individuals were examined. The average level of ROS in the patients' serum was 0.3062 ± 0.1539 and in the controls it was 0.1783 ± 0.0780. A significant increase in ROS levels was foundin patients when compared to healthy controls (Table), suggesting a linking role of free radicals in diabetes mellitus and MI complications. ROS levels among different parameters including sex, age, and hypertension were nonsignificant. Serum ROS concentrations in patients who smoked compared to normal subjects were significantly increased when compared using multivariate linear regression analysis (Table). These data suggest the important role of smoking in diabetes as well as in diabetic complications.

3.2. Antioxidant SOD activity

Serum SOD level, as an antioxidant, was measured. As shown in the Table and Figures 1a–1c, SOD activity was significantly decreased in diabetic patients with MI (45.53 ± 11.88 ng/mL) compared to healthy subjects (76.36 ± 10.931 ng/mL). SOD activity significantly decreased in diabetic patients, specifically in smokers.

3.3. Expression of calpain-1 in patients with type 2 diabetes and MI

The expression of calpain-1 in type 2 diabetes mellitus patients with MI as well as in healthy individuals was examined by immunoblotting (Figure 1). Comparatively, more calpain expression was observed on immunoblots from patients than from healthy individuals.

4. Discussion

MI commonly occurs when there is a rapid decline in coronary blood flow following a thrombotic occlusion of a coronary artery previously narrowed by atherosclerosis. Diabetes mellitus is one of the features that increase mortality in patients with acute MI (24). Many studies focused on and assessed different oxidative stress-related features in diabetes and MI individually, but there is a lack of studies addressing both issues in combination. The present study is based on analyzing the stress-responsive

Table. Multiple linear regression analysis of determinants ROS and SOD in type 2 diabetic patients with MI.

Variables	Patients (n = 100)	Standardized coefficients (beta)	P-value
ROS (absorbance)			
Patients/controls	0.3062/0.1783	0.430	<0.0001
Males/females	63%/37%	0.056	0.528
Age (<50/>50 years)	38%/62%	–0.042	0.634
Hypertension (HTN^+/HTN^-)	60%/40%	–0.009	0.924
Smokers/nonsmokers	72%/28%	0.550	<0.0001
SOD (ng/mL)			
Patients/controls	46.53/91.5	–0.797	<0.0001
Males/females	63%/37%	0.103	0.167
Age (<50/>50 years)	38%/62%	–0.009	0.902
Hypertension (HTN^+/HTN^-)	60%/40%	0.017	0.827
Smokers/nonsmokers	72%/28%	–0.682	<0.0001

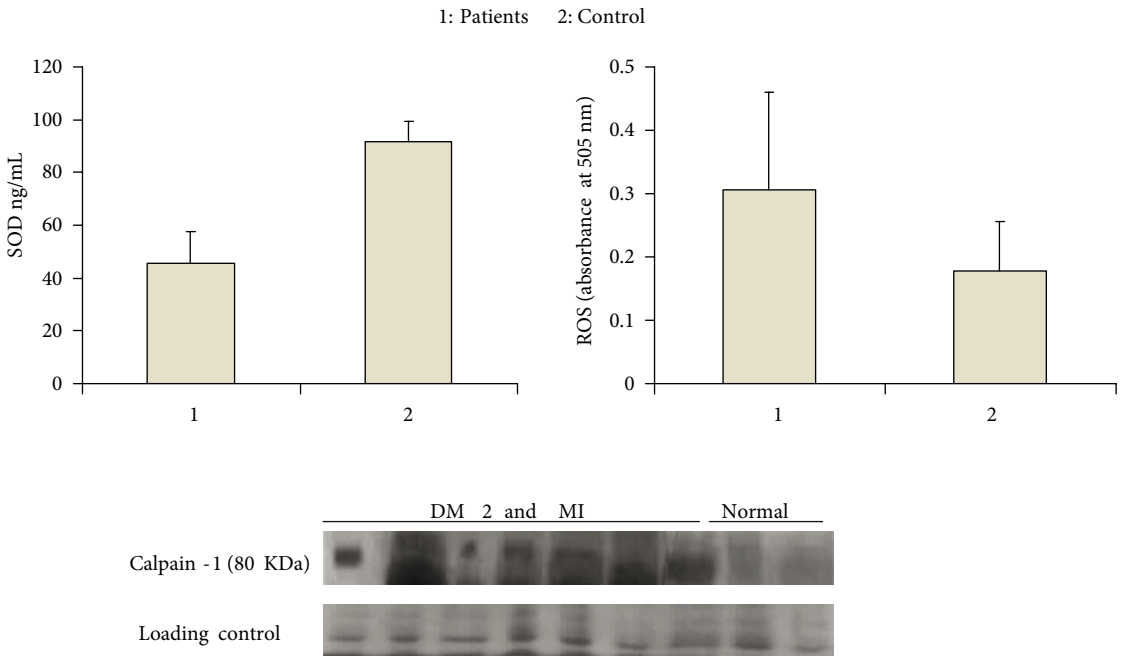

Figure 1. SOD, ROS, and calpain expression in diabetic and myocardial infarction patients. **a)** SOD activity in diabetic and MI patients (1) and normal individuals (2): reaction mixture containing L-methionine, NBT, and Triton was illuminated with a fluorescent lamp. After addition of riboflavin, SOD activity was analyzed in serum samples at 560 nm. **b)** ROS concentrations in diabetic and MI patients (1) and normal individuals (2). The DEPPD method was employed for detecting ROS at 505 nm, relating high absorbance rate with increased ROS concentrations. **c)** Blot analysis of calpain-1 in serum of diabetic MI patients. The 6 left-side lanes represent calpain expression in diseased samples and the extreme 2 right lanes represent normal healthy controls.

factors that in combination affect diabetes-linked MI and may be helpful for designing future specified therapies for diabetes-linked MI.

Previously a relationship between MI and oxidative stress, as well as altered concentrations of antioxidant enzymes, was documented (10). In the current

investigation, elevations of serum free radicals and downregulation of SOD in patients with type 2 diabetes and MI were observed when compared to normal subjects. Furthermore, smoking is known to have a significant positive association with the increase of production of free radicals by polymorph nuclear leukocytes (25). In the current study, increased ROS levels in patients with smoking habits are consistent with previous findings and suggest smoking as a contribution to diabetic complication via ROS regulation. The present study shows a significant increase of ROS to a certain extent, but the data of diabetic MI patients as compared with normal controls show that the increase in ROS is far greater. SOD activity was also observed to be significantly reduced in MI and diabetic patients, specifically in smokers, suggesting that increased oxidative stress in smokers renders reduced activity of the ROS scavenging machinery. Thus, we can conclude from the present study that DM patients who smoke may be more prone to MI than nonsmoking DM patients.

Previous studies indicated that calpain is of potential importance in the regulation of the proteolysis of key enzymes and structural proteins as well as proinflammatory responses (26,27). However, as of yet, there is no supporting study that indicates the role of calpain-1 in diabetes; only its regulation of myocardial hypertrophy has been revealed (28). Some studies suggested a possible increase in expression of calpain-10 in pancreatic cells during diabetes (29), but there is also a contradictory study showing a decreased expression of calpain-10 during diabetes (30). It is reported that in diabetes the calcium level increases (31) and that may also serve as an activation factor of calpain. Calpain and ROS both activate Bax and increase mitochondrial permeabilization and caspase release that ultimately play important roles in apoptosis (32). Bax was previously reported to have a basic role in the pathogenesis of myocardial infarction (33).

The results reported in this study show a high expression of calpain-1. The increase in ROS is also proportional to the increase in calpain. Calpain may be used as a possible efficient diagnostic marker for DM-linked MI. We infer that regulation of calpain-1 may, to some extent, play a role in the management of the disease, or in its treatment, as it also has a role in the apoptosis of cardiomyocytes (34). It is also hypothesized that an increase in calpain may result in apoptosis, which further leads to an increase in ROS due to mitochondrial membrane dysfunction.

Limitations of the present study include the need to understand in more detail the role of calpain-1. Its molecular regulation should be elucidated fully in patients with type 2 diabetes mellitus and MI. In the present study we were also unable to place antioxidants into an experimental model of diabetes and MI. Future studies must be carried out to address these issues.

In conclusion, elevated ROS levels are observed in type 2 diabetes mellitus patients with MI, whereas SOD levels are reduced. As a result there is an imbalance between oxidants and antioxidants that leads to the generation of free radicals, suggesting a possible link between these parameters and type 2 diabetes mellitus with MI. Supplementation with antioxidants may alleviate the production of ROS. Our study clearly showed stress-responsive factor calpain-1 expression in the serum of patients. Further research should be carried out in this new area of coronary heart diseases studies. The current study presents new avenues for designing future specified therapeutic interventions for type 2 diabetes patients with MI.

Acknowledgments

Part of this research work was supported by a TWAS-COMSTECH research grant and by the Higher Education Commission of Pakistan.

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