Int J Legal Med (1997) 110:1–4

ORIGINAL ARTICLE

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Antioxidant-related enzymes in myocardial zones and human pericardial fluid in relation to the cause of death

Received: 6 July 1995 / Received in revised form: 1 July 1996

Abstract The aim of this work was to shed light on hypoxic and ischemic processes in the heart that may lead to irreversible or lethal myocardial injury. We determined malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities in human cardiac tissues from 45 medico-legal autopsies of persons who died from different causes. Samples were taken from three different areas of myocardium: the anterior and posterior walls of the left ventricle, and the interventricular septum. We used light microscopy to examine the heart sections (hematoxylin-eosin and Masson's trichromic stains), and studied the K^+/Na^+ ratio and pericardial fluid. A decrease in GSH-Px activity was found in cases with severe atherosclerosis of the coronary artery in comparison with the group with slight or moderate atherosclerosis. Postmortem activities of GSH-Px and SOD were significantly different in the three myocaridal zones studied. An increase in GSH-Px activity in the interventricular septum was noted in cases of cardiac deaths. Antioxidant-related enzymes such as GSH-Px and SOD can therefore be regarded as new biochemical markers indicative of myocardial hypoxia. The possible applications to the postmortem diagnosis of the cause of death are discussed.

Key words Postmortem changes · Malondialdehyde · Antioxidant-related exzymes · Cause of death

Introduction

The precise cause of death can sometimes be difficult to establish in forensic practice. There have been many attempts from different perspectives to find reliable markers of hypoxic/ischemic myocardial damage. Many morphological, histochemical, enzyme-histochemical, and biochemical methods have been used in attempts to diagnose myocardial damage and especially for the exact diagnosis in cases of early myocardial infarction [5, 16]. The usefulness of conventional histology is limited because myocardial damage is usually manifested after 5–8 h. The results of enzyme-histochemical techniques are difficult to interpret because of artifacts caused by autolysis. Most available methods are nonspecific, or the changes induced by ischemia occur too late [14]. Biochemical methods include analysis of the pericardial fluid [19, 23] as a useful source of information on the state of the myocardium, while other researchers have used cardiac tissue itself [19]. Although no definitive solutions have been found to this problem, biochemical methods have nevertheless shown themselves to be particularly useful for demonstrating myocardial damage at earlier stages than is possible with histological methods.

There is currently evidence that several clinical situations which result in tissue injury do so by generating toxic metabolites of oxygen in amounts exceeding the capacity of tissue to protect itself [13, 26]. Included in these pathological conditions are myocardial infarction, cardiac arrhythmias, alcoholic myocardiopathy, and coronary artery disease [6, 28]. Free radicals can injure cells and tissues [15] producing, among other effects, lipid peroxidation of membranes including the production of malondialdehyde. In mammals, glutathione peroxidase (GSH-Px; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) are primarily involved in protecting cellular structures against peroxides and free radicals [10, 22, 36]. Imbalances caused by the excessive production of radicals or a decrease in defense systems result in varying degrees of cellular injury and may even cause cell death. Although much research has been devoted to free radicals in human pathology, studies that can be applied to cardiac forensic pathology are lacking. We have attempted to determine the usefulness of the postmortem analysis of two cellular antioxidant enzymatic systems (GSH-Px and SOD) in caridac tissue and pericardial fluid in cases of death re-

Preliminary results of this work were presented at the XVIth Congress of the International Academy of Legal Medicine and Social Medicine, Strasbourg (France), 1994

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lated to hypoxic/ischemic mechanisms that affect the heart, as well as in other causes of death. Malondialdehyde (MDA) an indicator of the extent of lipid peroxidation, was also measured in cardiac tissue from the same samples.

The usefulness of biochemical postmortem analysis of these parameters in establishing the postmortem interval has recently been reported [32]. No changes were detected in GSH-Px or SOD activities nor in MDA concentration in relation to the postmortem interval, which means that these parameters may be useful in postmortem studies to diagnose the cause of death. The aim of this work was to shed light on hypoxic and ischemic processes in the heart that may lead to irreversible or lethal myocaridal injury. We hypothesize that myocardial oxidant stress generated in some hypoxic myocardial antemortem situations can be measured or detected postmortem.

Material and methods

The material comprised a total of 45 cadavers (8 females and 37 males) autopsied at the Instituto Anatomico Forense of Malaga (Spain) with known times of death. Corpses were maintained at 4° C until the autopsies were performed at time intervals ranging between 6 and 24 h after death. The mean age of the deceased was 43 years (± 22). The heart and pericardial fluid were removed in each case. Samples of pericardial fluid were centrifuged at 1000 g for 10 min to eliminate blood contamination, and the supernatant was frozen at –40° C for biochemical analysis. Hearts were dissected using the transverse slicing method [16, 19], but before slicing the heart, coronary arteries were first dissected by serial transverse sectioning. The areas of maximal narrowing were noted by specifying the degrees of reduction of the cross-sectional area of the lumen (e.g. 0–25% = slight atherosclerosis; 26–75% = moderate atherosclerois, and > 75% = severe artherosclerosis) [34]. Based on information from previous studies, only three specific sites of the myocardium were selected for analysis: the anterior wall of the left ventricle, the posterior wall of the left ventricle, and the interventricular septum [16, 19]. Samples for biochemical analysis were taken from the intermediate ventricular slice and then frozen and kept at –40° C. Samples were stored for a maximum period of 6 months. Microscopical examination was performed on samples taken from the ventricular slice immediately underlying the intermediate one, and were kept in 10% formaldehyde until the studies were carried out.

The morphological examination consisted of a macroscopical (autopsy) and histological examination of heart sections stained with hematoxylin-eosin and Masson's trichromic stains. The K^+/Na^+ ratio was determined in myocardial tissue using a modification of methods described elsewhere [16, 19, 31].

Biochemical analyses of both myocardial tissue and pericardial fluid were performed. We measured GSH-Px and SOD activities, and lipid peroxidation was also determined in heart samples. Based on information from previous studies [9, 25] and on work carried out in our laboratory [32], no significant changes in any of these parameters should occur under the storage conditions used. Tissue samples consisted of 0.5 g of epicardium plus endocardium from each of the three specific sites. To measured enzyme activity the samples were homogenized according to Marklund [24] in 100 vols of 10 mM phosphate buffer, 30 mM KCl, adjusted to pH 7.4. This homogenate was sonicated for 5 min and then centrifuged at 2500 g for 10 min, and the supernatants were used for enzyme analyses and protein determination. GSH-Px activity was measured according to Paglia and Valentine [29], using t-butyl hydroperoxide as the substrate and adding 20 mM sodium azide to the reaction mixture to inhibit catalase activity. Blank values obtained without the addition of samples were subtracted from assay values. The mixture components, with the exception of hydroperoxide, were preincubated at 37° C for at least 2 min before the reaction was started. Linear rates of NADPH oxidation were recorded spectrophotometrically (340 nm) at 25° C. SOD activity was measured according to the method of Crapo et al. [11], based on the capacity of SOD to inhibit the reduction of ferricytochrome c by xanthine oxidase. Sufficient xanthine oxidase was added to the reaction mixture to catalyse an absorbancy change of 0.025/min at 550 nm. Calculations were based on a standard SOD curve at various final concentrations (0.1–1 U SOD/ml, Sigma S-7008) run under the same specified optimal assay conditions. When the results of enymatic analyses were expressed as specific activity, proteins were quantified in all samples.

The extent of lipid peroxidation in postmortem tissues was estimated by a thiobarbituric acid (TBA) test for malondialdehyde (MDA). Samples were homogenized according to Yoshida et al. [35] in 10 vol physiological saline containing 1 µg BHT (2,6-ditert-butyl-p-cresol) dissolved in 10 µl methanol. BHT was used to prevent artifactual oxidation of polyunsaturated free fatty acids during the assay. Thiobarbituric acid reactants were assayed using a technique modified from Kogure et al. [18]. An aliquot of the homogenate (200 µl) was solubilized with 100 µl of 7% sodium dodecyl sulfate solution. To this mixture we added 800 µl 0.1 M HCl and 400 µl 0.5% aqueous solution of TBA. The reaction mixture was heated for 60 min in boiling water. After cooling, 2 ml of N-butanol was added, followed by mixing for 15 s and centrifugation at 1100 g for 10 min. An aliquot of the organic layer was then taken for fluorimetric determination (excitation at 532 nm, emission at 553 nm) [4]. Malondialdehyde was estimated using a standard curve of 1,1,3,3-tetraethoxypropane (from 0.05–1 nmol/ml) according to Placer et al. [30].

Cases were grouped in relation to the cause of death according to their physiopathological similarity [20]:

Group 1: Cardiac deaths	7 cases
Group 2: Craniocerebral trauma	11 cases
Group 3: Multiple trauma	8 cases
Group 4: Mechanical asphyxia	5 cases
Group 5: Other violent deaths	5 cases
Group 6: Other natural deaths	9 cases

Group 1 consisted of 5 cases of acute myocardial infarction and 2 cases of congestive heart failure. All cases included in this group fulfilled the following two criteria: K^+/Na^+ ratio equal to or less than 0.8 in at least two of the three different areas of the myocardium, and clear signs of myocardial injury on both macroscopic and microscopic examination. None of these criteria were satisfied in any of the cases included in other groups of causes of death.

Statistical analyses consisted of one-way (ANOVA I) and two-way (ANOVA II) analyses of variance.

Results and discussion

It is currently accepted that differences in cardiac metabolism exist between zones of the heart. Lachica et al. [21] studied the levels of fatty acids, lipids, and carnitine in the zones of cardiac muscle, and detected significant metabolis differences that were dependent on muscle contractile activity in different cardiac structures. Apart from this study, there has been little information from postmortem studies on the different metabolic regions of the human cardiac muscle [17]. We investigated the differences in glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA) between myocardial zones. Differences in these enzymes according to the cause of death is another area of interest. We found no differences in any of the parameters studied between the six causes of death (results not shown). When cases were

Table 1 Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities in different zones of the myocardium in relation to the cause of death

Myocardial zones	GSH-Px		SOD	
	Cause of death		Cause of death	
	Cardiac(1)*	Noncardiac(2)	Cardiac(1)**	Noncardiac(2)
Anterior wall left ventricle	3.5 ± 1.1	3.0 ± 1.0	77.2 ± 16.1	77.3 ± 12.7
Posterior wall left ventricle	2.7 ± 1.03	2.8 ± 0.9	69.9 ± 15.3	68.6 ± 12.4
Interventricular septum	3.9 ± 1.5	2.9 ± 0.9	75.3 ± 15.1	72.3 ± 13.2

Values are expressed in U/g of tissue and represent the mean ± S.D. of: (1) $n = 7$ cases, and (2) $n = 38$ cases. Significant differences between zones of the myocardium (*) $F_{exp} = 6.74$; 2 d.f.; $P \leq 0.01$; (**) $F_{exp} = 3.43$; 2 d.f.; $P \leq 0.05$

grouped as cardiac deaths (group 1) or noncardiac deaths (the rest of groups of causes of death), significant differences in both GSH-Px and SOD activities were found in the three myocardial zones (Table 1). However, no significant differences were found for MDA concentrations in different areas of the myocardium. The average MDA concentration for these three myocardial zones studied were: 1.02 nmol MDA/g tissue for the anterior wall of the left ventricle; 1.19 nmol MDA/g tissue for the posterior wall of the left ventricle and 0.91 for the interventricular septum.

Glutathione peroxidase activities were higher in the interventricular septum in the group of cardiac deaths than in the other myocaridal zones in the group of noncardiac deaths. Different clinical situations (acute myocardial infarction, cardiac arrhythmias, coronary artery disease, etc.) may give rise to myocaridal hypoxic injury, resulting in the excessive release of hydroperoxides [6, 28]. Glutathione peroxidase is one of the main reducers of hydroperoxides produced by cells [36]. Experiments in rats hearts have shown an increase in GSH-Px activity in myocardial ischemic processes [3]. However, in their study of fatty acids in human cardiac muscle in relation to the cause of death, Lachica et al. [20] found the lowest levels in cases of death by myocardial infarction. The increase we detected in GSH-Px activity in the interventricular septum may reflect the need to degrade excess hydroperoxides presumably produced during myocardial hypoxia. For SOD, significantly lower activities were found in the posterior wall of the left ventricle when compared to the other myocardial zones in the group of cardiac deaths. Moreover, the SOD activity was higher in the anterior wall of the left ventricle and in the interventricular septum, which are areas with a higher frequency of ischemic myocardial damage [8].

These regional variations suggest the existence of a different antioxidant metabolism in each zone according to their metabolic and functional requirements [2]. Our results confirm those of Lachica et al. [21], who detected higher levels of free fatty acids in the left ventricle. The rates of antioxidant-related enzymes found might therefore be a logical response to the need for continuous action by these enzymes in response to elevated levels of fatty acids.

Enzymatic activities of GSH-Px and SOD were measured in postmortem pericardial fluid and then related to the cause of death. No differences were found between the causes of death and these biochemical parameters (results not shown). Free radicals produce local effect in areas of tissue ischemia, and then propagate to adjacent zones via chain reactions [36], therefore a certain period of time has to elapse for the effects to be noted in areas further from the original source.

It has now been accepted that the processes of ischemia and subsequent reperfusion in the myocardium increase the generation of free radicals from different sources (catecholamines, neutrophiles, xantine oxidase, etc.) [1, 12]. The proper functioning of the cardiac muscle requires adequate vascularization so that changes in the physiological metabolic demand can be met largely by increases and decreases in the coronary blood flow [33]. If for any reason (the most frequent being perhaps coronary artheromatosis) the supply of oxygen to the myocardium becomes restricted, the result is hypoxic/ischemic damage of varying degree depending on the vessel affected and the length of time the process lasts. We investigated the changes in antioxidant-related enzymes in relation to the degree of atherosclerosis. When we compared GSH-Px and SOD activities and MDA concentrations in different myocardial zones according to the severity of atherosclerosis in coronary arteries, no differences were found between the anterior and posterior wall of left ventricle. Nevertheless, significant difference were found for GSH-Px in the interventricular septum (Table 2): GSH-Px activity was higher in cases with slight or moderate atherosclerosis when compared to cases with severe atherosclerosis, regardless of

Table 2 Glutathione peroxidase activity in interventricular septum in relation to the severity of coronary atherosclerosis in different causes of deaths

Cause of death	Severity of atherosclerosis	
	I + II	III
Cardiac deaths	4.84 ± 1.05* (4)	2.73 ± 1.20 (3)
Non cardiac deaths	2.98 ± 0.90* (36)	2.72 ± 1.14 (2)

Values are expressed in U/g of tissue, and represent the mean ± S.D. of the cases in parentheses. Significant differences with group III, * $P \leq 0.05$. I: slight atherosclerosis; II: moderate atherosclerosis; III: severe atherosclerosis

whether the cause of death was considered to be cardiac or noncardiac.

In summary, we found metabolic differences in the antioxidant enzymatic systems between different zones of the myocardium. These differences may enhance our knowledge of cardiac metabolism. The GSH-Px and SOD enzymatic systems are undoubtedly involved in the hypoxic processes that can lead to lethal post-ischemic injury of the myocaridum. This suggests that these two postmortem biochemical markers are indicative of the presence of a myocardial hypoxic pathology. Therefore, these data are of interest to the field of forensic medicine and particularly to the postmortem diagnosis of the cause of death.

Acknowledgement We thank Karen Shashok for improving the English style of the manuscript

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