In the present study, we assessed oxidative stress in patients with dilated cardiomyopathy of ischemic or idiopathic etiology. For this reason we measured whole blood reduced glutathione, erythrocyte superoxide dismutase, susceptibility of erythrocyte membranes and erythrocytes to peroxidation, and SH content of erythrocyte membranes in 12 patients (8 men and 4 women, ages 31 to 66 years) with idiopathic dilated cardiomyopathy, in 11 patients (8 men and 3 women, ages 32 to 65 years) with ischemic dilated cardiomyopathy, and in 21 healthy volunteers (12 men and 9 women, ages 25 to 67 years). There was no statistically significant difference between the two patient groups for the indicators studied (P > 0.05). Blood glutathione, erythrocyte superoxide dismutase, and membrane SH content of both groups of patients was decreased compared with controls (P < 0.05), whereas erythrocyte and membrane susceptibility to peroxidation were increased (P < 0.05). We conclude that patients with idiopathic or ischemic dilated cardiomyopathy exhibit abnormalities of a range of markers of increased oxidative stress. These abnormalities may contribute to contractile dysfunction, increased incidence of fatal arrhythmias, and sudden death.

Reactive oxygen species (ROS) are constantly formed in the human body and removed by an antioxidant defense system. In healthy individuals, the generation of ROS appears to be approximately in balance with antioxidant defense. An imbalance between ROS and antioxidant defenses in favor of the former has been described as oxidative stress. In some human diseases, increased oxidative stress may make an important contribution to disease pathology. However, increased oxidative stress may be a result of disease pathology [1, 2].

Patients with myocardial failure often die suddenly of arrhythmias. Patients in the New York Heart Association’s (NYHA) Class III or Class IV have an overall yearly mortality of 40% to 60% [3]. ROS may play important role in the sudden death of these patients, and increased ROS generation has been described in patients with congestive heart failure [4–7]. However, congestive heart failure is an ill-defined syndrome. Therefore, it may be of interest to study patients with congestive heart failure secondary to conditions such as cardiomyopathy. Patients with cardiomyopathy have been reported to have decreased serum selenium [8]. In the Keshan area of China, cardiomyopathy is endemic (Keshan disease) among children under 15 years of age and is associated with selenium deficiency and controlled by selenium supplementation. Selenium is necessary for the activity of glutathione peroxidase (EC 1.11.1.9), a member of the antioxidant system. Drugs such as daunomycin and doxorubicin may also lead to cardiomyopathy through increased ROS generation [9].

Peroxidative damage to cellular constituents such as membrane lipids and proteins is the major threat in conditions with increased oxidative stress [10]. Increased ROS generation may depress myocardial contraction through interaction with membrane lipids or proteins [11]. Blood can reflect the lability of the whole body to oxidative conditions. In experiments in which oxidative stress is evaluated, erythrocytes appear to be excellent material because of their easy availability, their simple structure, and relatively large amounts of polyunsaturated fatty acids in their membranes. Particularly, the lability of erythrocyte membranes to oxidative stress in vitro may reflect the lability of other cell membranes to oxidative damage in vivo [12].

Although several studies have reported evidence of increased oxidative stress and lipid peroxidation in pa-
There was obstruction before the starting point of the study. In ISCDC subgroup, patients underwent coronary angiography in the last year, diagnostic picture indicating dilated cardiomyopathy. All patients had a clinical and two-dimensional echocardiographic parameter was entirely characteristic for enoximone-resistant, rheumatic, congenital, infectious, or pericardial disease; valvular heart disease; mitral valve prolapse; or hypertrophic cardiomyopathy. All patients were idiopathic dilated cardiomyopathy in 12 patients (8 men and 3 women, ages 32 to 65 years). No patient who had clinical evidence of hypertension, rheumatic, congenital, infectious, or pericardial disease; valvular heart disease; mitral valve prolapse; or hypertrophic cardiomyopathy were included in the study. All patients had a clinical and two-dimensional echocardiographic picture indicating dilated cardiomyopathy. All patients underwent coronary angiography in the last year before the starting point of the study. In ISCDC subgroup, there was obstruction >70% in one or more of epicardial coronary arteries. The patients who had none or <50% obstruction were included in the IDC subgroup. Physical examination and anamnesis, routine radiological examination (plain chest radiography), biochemical (glucose, urea, creatinine, calcium, inorganic phosphorus, free triiodothyronine, free thyroxine, thyrotropin, iron and iron-binding capacity, and ferritin in serum), and hematologic (hemogram and erythrocyte sedimentation rate) tests were carried out in all patients. No patient had clinical signs of acute ischemia or angina. Although no electrocardiographic parameter was entirely characteristic for the patients under study, electrocardiograms obtained for all patients were abnormal in every case. In the patients from IDC subgroup, there was no known underlying cause (alcohol or drug abuse, cytotoxic agents, viral infections, asthma or atopic diseases, hemochromatosis, sarcoidosis, or acute or chronic myocarditis) that could lead to dilated cardiomyopathy, and these subjects were categorized as patients with IDC. Clinical severity was assessed according to the NYHA classifications III and IV. The left ventricular ejection fractions of these patients were <35%. All the patients were receiving conventional medication including digitalis, diuretics, angiotensin-converting enzyme inhibitors, and nitrates. No patient received calcium antagonists and thiol-containing agents such as captopril or etacrinic acid. We also included 21 healthy asymptomatic subjects, 12 men and 9 women, ages 25 to 67 years, in this study.

Materials

Chemicals. Albumin (human, fraction V), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium, sodium dodecyl sulfate (SDS), SOD (from human erythrocytes), 1,1,3,3-tetraethoxypropane, thiobarbituric acid (TBA), and xanthine were purchased from Sigma; phenylhydrazine hydrochloride, metaphosphoric acid (MPA), phosphotungstic acid, and sodium azide were from Riedel; n-butanol, Tris, CuSO₄ · 5 H₂O, CuCl₂, EDTA- Na₂, NaCl, HCl, K₂HPO₄, KH₂PO₄, hydrogen peroxide 35%, and H₂SO₄ were from Merck; xanthine oxidase was from Boehringer Mannheim. All chemicals and solvents were analytical reagent grade and were used without further purification.

Apparatus. Membrane isolation was performed with a Sorvall® RC M120 EX microultracentrifuge (rotor no. RP 80AT) (DuPont, Sorvall Products); all other centrifugation procedures were performed with Heraeus Megafuge 2.0 RS and Heraeus Sepatech Minifuge T centrifuges; TBARS were measured by a Jasco FP-777 spectrofluorometer (Japan Spectroscopic Co.); spectrophotometric measurement of GSH was performed by a Shimadzu UV-1201 spectrophotometer (Shimadzu Corp.); readings of erythrocyte SOD activity and erythrocyte membrane protein were performed by a Vitatron SPS spectrophotometer (Vital Scientific); hemoglobin (Hb) measurements in whole blood and erythrocyte hemolysates were performed by a Coulter Counter S Plus VI (Coulter Electronics).

Blood collection. Venesection of the patients took place on the morning of the second day of hospitalization. Venous blood samples (5 mL) were taken from the antecubital vein into ice-chilled Vacutainer Tubes (Becton Dickinson) containing EDTA as anticoagulant and processed immediately. Venesection and sample processing in the control group were performed under similar conditions to the patient group.

Procedures

GSH. As soon as the blood samples reached the laboratory, 200 μL of whole blood was assayed immediately for GSH (practically erythrocyte GSH) by the method of Beutler et al. [13], which involved MPA extracts and DTNB. Hb content of the remaining whole blood was measured by a Coulter Counter and GSH content was calculated as micromoles of GSH per gram of Hb.
Preparation of packed erythrocytes. Blood samples were centrifuged immediately at 3000g for 5 min at 4°C. Plasma and Buffy coat were removed by careful suction and the cells were resuspended in 5 mL of 9 g/L NaCl solution. After mixing well by inversion, the sample was centrifuged again at 1500g for 5 min at 4°C. This washing procedure was repeated twice more. Packed erythrocytes prepared in this way were used for membrane isolation, erythrocyte susceptibility to peroxidation, and erythrocyte SOD activity measurements.

Membrane isolation. Erythrocyte membranes were isolated according to Hanahan and Ekholm [14]. However, we slightly modified this method: In brief, washed erythrocytes were suspended in isotonic Tris buffer pH 7.6 to an approximate hematocrit of 50% (by adding ~2 volumes of Tris buffer to 1 volume of packed cells), and the cells were resuspended by gentle inversion. Two aliquots of 500 μL of this suspension were transferred into two different 4.0-mL polycarbonate microcentrifuge tubes. Ice-chilled hypotonic Tris buffer pH 7.6 containing 5 mmol/L EDTA, 3.0 mL, was added forcefully into the cell suspension. The tubes were allowed to stand for 5 min, mixed by inversion, and then centrifuged at 20 000g for 10 min. After centrifugation, the supernatant was removed without losing membranes by careful suction; the pellet was resuspended by adding 3.0 mL of hypotonic Tris buffer pH 7.6 without EDTA, the tubes were centrifuged again at 20 000g for 5 min, and the supernatant was removed by suction. The last procedure was repeated twice more. Finally, membranes in two different tubes were combined in 1.0 mL of hypotonic Tris buffer. These membrane samples were analyzed for membrane susceptibility to peroxidation and for measurements of SH contents of membranes. Protein measurements in these preparations were also carried out by a modification of the Lowry procedure [15]. Membranes were solubilized before measurements by adding 20 g/L SDS as final concentration.

Membrane susceptibility to peroxidation. Susceptibility to in vitro lipid peroxidation of erythrocyte membranes was determined according to Taus et al. by incubating membrane samples with 0.3 mmol/L phenylhydrazine in a 300 mL/L methanol solution at 37°C for 45 min [16]. Then TBARS were determined in these samples by the method of Wasowicz et al. [17]. Results were calculated as nanomoles of MDA per milligram of membrane protein.

SH content of membranes. Free SH groups in the membrane preparations were determined according to the method of Habeeb [18]. In brief, 100 μL of membrane sample was added to 900 μL of Tris-EDTA buffer, pH 8.2. Absorbance was measured at 412 nm after adding 20 μL of 10 mmol/L DTNB (in 0.05 mol/L phosphate buffer, pH 7.4). After subtraction of absorbances of appropriately prepared reagent and sample blanks, SH concentrations were calculated by the use of molar absorptivity of thionitrobenzoate anion (ε412 = 13 600) and the results were expressed as micromoles of SH per milligrams of membrane protein.

Erythrocyte SOD. The Cu,Zn-SOD content of erythrocytes was determined through the inhibition of nitroblue tetrazolium reduction by the enzyme in the xanthine–xanthine oxidase system according to Sun et al. [19]. The calibration curve was constructed by using human erythrocyte SOD as the calibrator, and results were calculated as milligrams of SOD per liter of erythrocytes.

Erythrocyte susceptibility to peroxidation. Erythrocytes were oxidized by hydrogen peroxide in the presence of azide according to Stocks et al. [20] and TBARS were measured by the method of Yagi [21]. Hb concentration of erythrocyte lysates was determined in the Coulter Counter and the susceptibility to peroxidation was calculated as nanomoles of TBARS per gram of Hb.

Statistical analysis
Data were calculated on a “SPSS for Windows” program. Results were expressed as means ± SEM. Equality of variances was controlled by Levene’s test. Differences between the groups were estimated, after checking the normal distribution, by means of independent Student’s t-test, or by Mann–Whitney U-test. A P-value <0.05 was considered significant.

Results
Figure 1 shows the distributions (dot diagrams) of membrane SH concentrations and susceptibility of membranes to peroxidation in the two groups of patients and the control group. Susceptibility of erythrocyte membranes to peroxidation was 0.598 ± 0.05 nmol TBARS/mg protein in the ISCDC group, 0.624 ± 0.07 nmol TBARS/mg protein in the IDC group, and 0.394 ± 0.03 nmol TBARS/mg protein in the control group; the values of the two patient groups were significantly different from those of the control group (P = 0.0002 in both). SH content of erythrocyte membranes of the ISCDC (141 ± 4.35 μmol/mg protein) and IDC (149 ± 5.06 μmol/mg protein) groups was significantly decreased compared with healthy controls (161 ± 2.58 μmol/mg protein, P = 0.0008 and P = 0.0173, respectively).

The distributions of blood GSH, SOD content, and susceptibility to peroxidation of erythrocytes are shown in Fig. 2. GSH contents of blood in the ISCDC (4.00 ± 0.34 μmol/g Hb) and IDC (4.07 ± 0.27 μmol/g Hb) groups were decreased significantly compared with the control group (5.16 ± 0.16 μmol/g Hb, P = 0.0003 and P = 0.001, respectively). In a similar way, SOD content of erythrocytes in the ISCDC (218 ± 20 mg SOD/L erythrocytes) and IDC (200 ± 21 mg SOD/L erythrocytes) groups were also lower than that in the control group (385 ± 23 mg SOD/L erythrocytes, P = 0.0001 and P <0.0001, respectively). However, susceptibility to peroxidation of patient erythrocytes was significantly higher than that of controls.
(743 ± 38 nmol TBARS/g Hb in the ISCDC group, 781 ± 29 nmol TBARS/g Hb in the IDC group, and 576 ± 13 nmol TBARS/g Hb in the control group; \( P = 0.0003 \) for ISCDC and \( P < 0.0001 \) for IDC).

On the other hand, although erythrocyte and membrane susceptibility to peroxidation of the IDC group was higher compared with the ISCDC group, these differences were not statistically significant (\( P > 0.05 \)). In similar way, even though slightly different erythrocyte SOD and membrane SH values existed between the two groups of patients, these differences were also statistically insignificant (\( P > 0.05 \)).

**Discussion**

In the present study, we determined increased oxidative stress in patients with dilated cardiomyopathy by proving that erythrocytes and erythrocyte membranes from these patients were more prone to lipid peroxidation and oxidative damage than those from healthy controls.

The protection of cells from damage by reactive oxygen species may be evaluated on the basis of some enzyme
activities at the cellular level. SOD, one of the very important intracellular antioxidant enzymes, is present in all aerobic cells and has an antioxidant effect against superoxide anion. Hydrogen peroxide, a reaction product of the superoxide dismutation reaction, inactivates SOD, and in the presence of hydrogen peroxide SOD acts as a prooxidant [22, 23]. It is well-known that SOD activity can be decreased by ischemia or hypoxia [24, 25]. In the present study we have shown that diluted cardiomyopathy, regardless of etiology, is associated with decreased SOD concentrations in erythrocytes. This result is consistent with that obtained by McMurray et al. [6].

GSH, the major nonprotein thiol in living cells, plays an important role as an antioxidant. It is pivotal in various protective systems such as glutathione peroxidase, glutathione transferase, and free radical reductase [22]. It is also responsible for the maintenance of protein thiol status in cells. GSH acts as a free radical scavenger and helps in regenerating other antioxidants; it is depleted during such reactions [26]. In the present study, whole-blood GSH concentrations of patient groups were decreased as compared with the control group. This result may be a consequence of increased GSH depletion due to increased oxidative stress in the patient group. Contrary to our results, McMurray et al. found in their study that patients with congestive heart failure had higher erythrocyte GSH concentrations than healthy controls [6]. They explained this situation by decreased erythrocyte SOD activity (if SOD activity is decreased then decreased production of hydrogen peroxide may lead to reduced oxidation of glutathione by glutathione peroxidase). However, intracellular hydrogen peroxide occurs not only by formation in the SOD reaction, but also by diffusing across cell membranes as rapidly as a water molecule [2]. Additionally, GSH may be depleted by organic peroxides through glutathione peroxidase activity, and certain GSH S-transferases can also catalyze such reactions; GSH reacts with disulfides enzymatically (through transhydrogenases) and nonenzymatically and so is oxidized to glutathione disulfide (GSSG); and GSH can react directly with free radicals or reactive electrophiles [26, 27].

These results show that antioxidant defense of erythrocytes in patients with dilated cardiomyopathy is weak compared with that in healthy controls. Thus, we found that susceptibility of erythrocyte lipids, particularly membrane lipids, to peroxidation under experimental oxidative stress was greatly increased in the patient group (TBARS in the two groups of patients were increased compared with controls after oxidant stress). Generally, TBARS measurement is a good index of lipid peroxidation. However, it may not be completely adequate to study the changes occurring in biological membranes because, in addition to membrane lipids, membrane proteins are also affected by oxidative stress. Therefore, thiol status of membranes may be a good indicator for the oxidative damage of membrane proteins. Sulfhydryl compounds, particularly GSH, have a weak S-H bond strength (=85 kcal/mol) and therefore are capable of repairing radicals formed in membranes such as carbon-centered radicals, which usually have bond strengths >90 kcal/mol [28]. In addition, many enzymes have SH groups in or near the active site. When peroxidation of membrane lipids is accelerated, lipid peroxidation products such as 4-hydroperoxynonenal and 4-hydroxy-2-alkenals both react with sulfhydryl groups of various enzymes and thus modify their activities. Thus some transport and receptor functions of various cell membranes are modulated by changes in redox status of protein-SH groups [29]. In the present study, we found that SH groups of erythrocyte membranes were decreased in both patient groups as compared with controls. This finding indicates that there is considerable membrane damage due to oxidative stress in patients with dilated cardiomyopathy.

Patients with dilated cardiomyopathy have impaired myocardial contractility and can have fatal ventricular arrhythmias. Membrane changes due to increased oxidative stress may be partly responsible for these manifestations of the disease, although ROS cannot be totally responsible for a particular disease. Peroxidation of membrane lipids is a relatively slow process. However, recurring ischemia–reperfusion cycles in the heart and skeletal muscle, and catecholamine autoxidation may increase membrane lipid peroxidation. But, the more important cause probably arises from changes in membrane proteins. In this respect, in vitro studies have shown that oxidation of SH groups due to oxidative stress can impair Ca\(^{2+}\)-ATPase activity of sarcoplasmic reticulum [30], and Na\(^+\) K\(^+\)-ATPase of plasma membranes [31]; the calcium release channel of the sarcoplasmic reticulum may also be impaired by ROS [32]. Moreover, hormone receptors involved in maintaining calcium homeostasis often contain a critical SH moiety [33]. These alterations may result in intracellular calcium overload and hence contribute to the mechanism of contractile dysfunction and ventricular arrhythmias in dilated cardiomyopathy. Thus increased oxidative stress → membrane changes → calcium overload could be a central vicious chain in these patients.

Another important aspect of SH oxidation is conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase (EC 1.1.3.22) exists in nonischemic, healthy cells predominantly as an NAD\(^+\)-dependent dehydrogenase [34]. This form of the enzyme uses NAD\(^+\) instead of molecular oxygen as the electron acceptor during oxidation of purines. But xanthine dehydrogenase is converted to xanthine oxidase by SH oxidation. This form of the enzyme has the ability to generate hydrogen peroxide and superoxide anion [35]. Release of these ROS during purine degradation may peroxidize the cell membrane [36]. Hisatome et al. demonstrated that purine degradation was increased in patients with congestive heart failure and there was the possibility of a relation between purine degradation and arrhythmia [37].

On the other hand, altered high-energy phosphate...
metabolism may contribute to the contractile dysfunction in patients with dilated cardiomyopathy. In a previous study, Hardy et al. demonstrated that myocardial high-energy phosphate concentrations were reduced in patients with dilated cardiomyopathy [38]. Creatine kinase (EC 2.7.3.2) plays an important role in myocardial and skeletal muscle high-energy phosphate metabolism. The creatine kinase reaction is essential for rapid resynthesis of ATP when the heart increases its work. Activity of this enzyme is also impaired as a result of SH group oxidation at the active site [39]. Hamman et al. [40] demonstrated that inhibition of the reaction of creatine kinase decreases the contractile reserve of the isolated rat heart. We think that creatine kinase activity is decreased—at least partly—via SH oxidation due to increased oxidative stress in patients with dilated cardiomyopathy.

In summary, our results suggest that increased oxidative stress is associated with dilated cardiomyopathy whether it is idiopathic or secondary to ischemic heart disease; membrane lipid peroxidation and protein damage are increased in these patients. It has been emphasized that a relative deficit in antioxidant reserves may contribute to cardiac failure [41]. Therefore, exogenous administration of antioxidants may slow the progression of cardiac abnormalities and may decrease the incidence of life-threatening ventricular arrhythmias or sudden death in dilated cardiomyopathy or, generally, in congestive heart failure.

We thank Gülten Kartal for help in venesection. This study was supported by Ankara University, Research Fund, with a code number of 93.30.00.19.

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