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SHORT COMMUNICATION

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A period of anaerobic exercise increases the accumulation of reactive carbonyl derivatives in the lungs of rats

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Abstract It is known that acute physical exercise may have diverse pathophysiological consequences in various organs due to free radical formation. We have investigated whether a period of anaerobic running to exhaustion in rats results in oxidative modification of proteins in the lungs. Six rats of an exercised group (E) ran for two periods of 5 min at a speed of 30 m·min^{-1} followed by a recovery period of 5 min, and then by a third period of running to exhaustion. Reactive carbonyl derivatives (RCD) were measured by the Western blot technique on lungs of E and control (C) rats. In addition, the activity of glutamine synthetase (GS) was also monitored as marker of oxidative damage to proteins. This investigation revealed significant exercise-induced increases in accumulation of RCD in the lungs of the E group compared with the C group. The RCD signals were visibly stronger in proteins with molecular weight of 55 kDa and 32 kDa. The activity of GS was higher by about 30% in E rats than in C rats. The present data suggest that anaerobic exercise induces protein oxidation in the lungs.

Key words Exercise · Protein oxidation · Lung · Glutamine synthetase

Introduction

Free radicals are formed in normal physiological processes. The physiological reactivity of these radicals protects cells against foreign bodies, regulates certain cell functions, and/or damages cell components. The massive increase in oxygen uptake that occurs in skeletal muscle in exercise is associated with increase in free radical formation as measured by electron paramagnetic resonance (Davies et al. 1982). Accordingly, it is known that a single bout of exercise maybe associated with an oxidative damage to a variety of organs (Davies et al. 1982; Rajguru et al. 1994; Radak et al. 1995). The oxidative stress-related consequences of exercise on the lungs is unclear. However, as in the lungs gas exchange occurs, this organ could be one of the first targets of free radical species.

During high intensity exercise, when the oxygen demand exceeds the oxygen supply, adenylate kinase activity is increased (Hellsten et al. 1989), which might be expected to lead to a significant increase of xanthine oxidase (XO) activity in the circulation (Hellsten et al. 1989; Radak et al. 1995). During such exercise oxygen is present, albeit limited amounts and thus there is a probability that the during this period and particularly during the recovery period the interaction of XO and oxygen results in oxygen-radical formation. Indeed, Alessio et al. (1988) have reported that high intensity exercise (1 min run at a speed of 45 m·min^{-1}) leads to much greater lipid peroxidation than exercise of a moderate intensity. Moreover, Radak et al. (1995) reported that there is a positive relationship between the level of lactate and XO activity in the blood following anaerobic exercise. Therefore, we hypothesize that there are some common characteristics between anaerobic interval exercise and ischemia/reperfusion. Since ischemia-reperfusion also promotes the conversion of xanthine dehydrogenase (XD) to XO resulting in oxidative damage of lungs (Moldeaus 1994), we propose that anaerobic exercise causes oxidative damage to the lungs.

Measurement of protein oxidation may be a very sensitive marker of oxidative stress (Radak et al. 1997) since radical species result in a variety of modifications in amino acid residues like cysteine, methionine, tryptophan, arginine, lysine, and histidine (Stadtman 1992). Among these, reactive carbonyl derivatives (RCD) are formed and they can be detected by their reaction with 2,4-dinitrophenyl hydrazine (DNPH) (Nakamura and Goto 1996).

The purpose of this investigation was to study the effects of a period of anaerobic exercise on protein oxidation in the lungs. In addition, the activity of glutamine synthetase (GS) was monitored, as it is easily modified by the attack of free radicals (Levine et al. 1981) and is often used as a marker of the effect of oxidative stress on proteins (Oliver et al. 1990). It was hypothesized that anaerobic exercise results in oxidative stress, which increases the accumulation of RCD and suppresses the activity of GS.

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Materials and methods

Animal care and exercise. Twelve 8-week-old male Wistar rats were obtained from Clea (Tokyo, Japan) and were cared for according to the Guid-

ing Principles for the Care and Use of Animals. The rats were assigned randomly to two groups, control (C) and acute exercise (E). The rats of E group were introduced to treadmill running with 6- to 8-min bouts of exercise at 12–26 $m \cdot min^{-1}$ for four days to accustom them to running. The treadmill was equipped with an electric shocking grid on the rear barrier to provide motivation for the animals. During the test the rats ran for two 5 min periods at the speed of 30 $m \cdot min^{-1}$ with the recovery interval of 5 min. Then, after a second recovery period of 5 min they exercised for greater than 5 min, the speed being increased by 3 $m \cdot min^{-1}$ until the rats reached exhaustion, i.e., when they were not able to run any further despite being shocked. The level of exhaustion was evaluated by the same criteria as described by Davies et al. (1982), Lew et al. (1988) and Radak et al (1995). Our pilot study demonstrated that this regime involves anaerobic metabolism as measured by lactic acid accumulation in blood.

Blood and tissue collection. Blood samples were taken from the tail vein of the E (2–3 min after the period of running to exhaustion) and C (rest) rats in order to measure lactic acid level (23L Yellow Springs Instruments lactic acid analyzer, Yellow Springs, OH, USA). The rats were killed by decapitation 1 h after the last exercise bout. This time period was allowed because it was shown that changes in RCD and activity of GS are more significant 1 h after oxidative stress than immediately after exercise (Oliver et al. 1990). The lungs were quickly removed, washed in cold saline to minimize the blood content in the tissue, then submerged in liquid nitrogen and stored at –80°C.

Biochemical analysis. The RCD was detected by immunoblot using monospecific antibodies against 2,4-dinitrophenyl hydrazones of oxidized bovine serum albumin as described by Nakamura and Goto (1996). In brief, proteins precipitated with trichloroacetic acid were suspended and incubated in a solution containing 10 mM DNPH and 2 N HCl for 1 h at 15°C. The resulting protein hydrazones were pelleted in a centrifuge at 11 000 × g for 5 min. The pellets were washed three times with ethanol-ethyl acetate (1:1) and then once with acetone. The final precipitates (1 mg protein) were dissolved in 1 ml buffer containing 8 M urea and 5% 2-mercaptoethanol using a sonicator for 10 min. Duplicate polyacrylamide gel electrophoresis of derivatized proteins was carried out in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Ten microgram protein/lane was loaded. After the electrophoresis the proteins were transferred to PVDF membranes. The membranes were then soaked in PBS containing 3% skim milk, 0.05% Tween and 0.05% sodium azide, and treated with anti-DNPH antibodies, prepared according to Nakamura and Goto (1996). After washing in buffer without antibodies, the membranes were treated with ^{125}I-Protein A (0.02 μCi/ml). Finally, the radioactive signals of each lane were quantified by BAS 2000 Bioimaging Analyzer (Fuji Film Co., Tokyo, Japan). The protein stain for each lane was quantified by a densitometer. The data are expressed as a protein stain density of each lane divided by radioactive density of antibody treated samples (Radak et al. 1997). Western blot analysis was carried out three times with three samples per group, and a randomly chosen data set is shown.

The activity of glutamine synthetase (GS) was measured by the method of Miller et al. (1978). The assay mixture was incubated at 37° for 60 min, it contained in 1.0 ml: 50 mM imidazole HCl, 50 mM NH_2OH, 100 mM L-glutamine, 25 mM potassium arsenate, 0.2 mM ADP, 0.5 mM $MnCl_2$ and samples with 500 µg protein content. Reactions were initiated by the addition of lung homogenate and terminated by 1.0 ml of 0.37 M $FeCl_3$/0.3 M trichloroacetic/0.6 M HCl. The precipitate was removed by centrifugation and the activity was determined at 505 nm. Parallel incubations of samples in assay mixture that lacked ADP were performed and served as controls. The protein concentration was determined by Pierce BCA methods. For GS assay, the results were reported as the mean of duplicate determination of six homogenate of lungs. The correlation between duplicate samples was r = 0.923.

Statistics. Data were analyzed using a two-way analysis of variance and Duncan's multiple range test. The level of significance was set at $P < 0.05$.

Results

The lactic acid concentration in the blood of E rats (9.7 ± 1.4 $mmol \cdot l^{-1}$) was significantly higher than that of the C group (1.3 ± 0.2 $mmol \cdot l^{-1}$). This result indicates that the rats were not able to obtain the sufficient amounts of oxygen during running, hence anaerobic metabolism supported a significant part of the running activity.

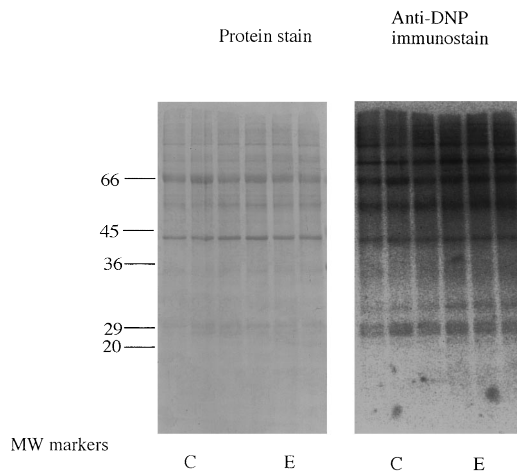

Fig. 1 Accumulation of reactive carbonyl derivatives (RCD) was more significant in lungs of exercised (E) than in control (C) rats. The signal density was measured by BAS 2000. The RCD accumulation was more visible in proteins with a molecular mass of ca 55 kDa and 32 kDa. Molecular mass markers are shown on the left of the Coomassie Blue stain panel

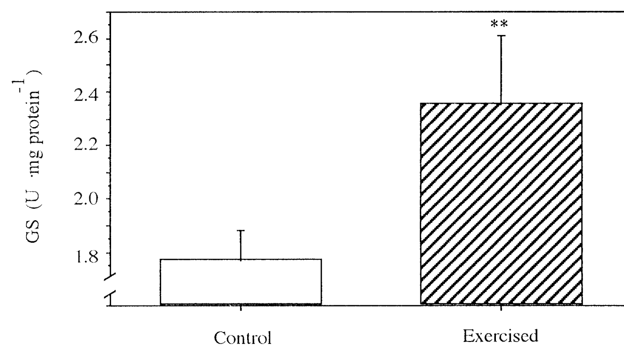

Fig. 2 The activity of glutamine synthetase (GS) was significantly ($P < 0.001$) higher in lung samples of exercised rats than control rats. Values are means ± SD

The accumulation of RCD in proteins of lungs was quantified as protein stain density divided by immunoblot radioactive density of each lane. Therefore, there is an inverse relationship between the numerical data and the accumulation of RCD. The summated protein density signals of each lane was divided by the summated signal density of RCD of the same lane. A significant difference ($P < 0.005$) in accumulation of RCD between E rats (3.53 ± 0.30 arbitrary units) and the C (4.87 ± 0.32 arbitrary units) group was measured (Fig. 1). The RCD signals were visibly stronger in proteins with molecular weight of 55 kDa and 32 kDa.The activity of GS, a possible marker of protein-related oxidative stress, increased significantly with exercise ($P < 0.001$), from 1.76 ± 0.11 U/mg protein to 2.36 ± 0.26 U/mg protein (Fig. 2).

Discussion

Oxidative modification of proteins is accompanied by the generation of protein RCD that can react with DNPH to form hydrazone derivatives (Oliver et al. 1990). The present study demonstrates for the first time that a single bout of exercise in-

creases the accumulation of RCD in lungs, so indicating a degree of oxidative stress. This in turn suggests that exercise-induced oxidative stress was not be completely curbed by the antioxidant system of the lungs. The radicals that generated the damage implied by RCD data could be formed from a variety of sources, such as by the electron transport chain of the mitochondrial respiration and by XO. During anaerobic exercise ATP synthesis involves the adenylate kinase reaction, resulting in the formation of hypoxanthine, which diffuses from the muscles and is further oxidized by XD on the surface of endothelial cells. The XD could be converted to XO by protease. The circulating XO so formed might result in oxidative damage at a site distant from its formation (Yokoyama et al.1990) as it reacts readily with molecular oxygen generating oxygen-radicals. The lungs, where the oxygen enters into the circulation, might be the first target of circulating XO-derived radicals, however this hypothesis needs verification.

The majority of investigators agree that XO-mediated free radicals are involved in the oxidative damage that is caused in lungs ischemia-reperfusion (Moldeaus et al. 1994). Under the current experimental conditions it seems unlikely that the lungs became ischemic. However, the XO that is formed by the inadequate supply of oxygen to exercising muscles might have generated damage in other organs (Yokoyama et al. 1990) including the lungs by causing an increase in the circulating level of XO.

Accumulation of RCD is mainly dependent upon the abundance of proteins as, in general, a stronger protein stain band is associated with a stronger band in immunoblot samples. In the present study, the RCD signals were visibly stronger in proteins with a molecular mass of 55 kDa and 32 kDa. This indicates that some proteins were more sensitive to the formation of RCDs under the present experimental condition than others and suggests that some forms of oxidative damage take place with selective order. This order might have physiological consequence, since the oxidative damage might be the first step in an adaptive process (Davies et al. 1982; Rjugaru et al. 1994). The identification of the damaged proteins generated in the lungs requires further research. However, one possible candidate is carbonic anhydrase, as it has a molecular weight of 31 kDa, and its residues are very sensitive to formation of RCD.

It is also possible that the increase in RCD was partly due to aldehydes derived from the lipid hydroperoxides that are formed during oxidative stress (Nakamura and Goto 1996). However, lipid peroxidation and protein oxidation are not inseparable processes, as they have different mechanisms (Radak et al. 1997). In spite of intensive washing of lung samples, we cannot completely rule out the possibility that the lung homogenate contained a certain amount of blood, and that this could have influenced the result we obtained for RCD. However, the pattern of protein stain of each sample was very similar, suggesting a high purity of lung homogenates; it seems unlikely that all samples contained the same amount of blood constituents with the same molecular weight.

The activity of GS of exercised rats was significantly elevated compared with that of the control rats. We do not know the time course of the alteration of GS, nevertheless the increases were significant 1 h after anaerobic exercise. The lung is a heterogeneous tissue; therefore, the activity of GS represents the mean enzyme activity of all the constitutive cells (Ardawi 1990). Glutamine (GLN), which is synthetised by GS, is a very important amino acid as it plays a crucial role in detoxifying ammonia. We do know that due to the adenylate kinase reaction anaerobic exercise increases the activity of AMP deaminase, so resulting in formation of ammonia. During exercise-induced acidosis, there is no change in blood GLN level to indicate increases in supply of GLN (Goldstein 1986). Therefore, GLN must be released from different tissues, possibly from skeletal muscle, liver, and lungs to detoxify ammonia (Ardawi 1990). Thus, the activity GS may be readily induced by anaerobic exercise in GLN releasing organs. The present study demonstrates that the activity of GS is increased in the lung 1 h after anaerobic interval exercise. However, at this time it is unclear whether the increase is due to the GLN release from the lung.

We report here that that anaerobic exercise simultaneously increases the accumulation of RCD in the lung and the activity of GS. This observation indicates that the accumulation of RCD is not always accompanied by the decreases in the activity of GS, as it occurs with aging (Stadtman 1992) or ischemia/reperfusion (Oliver et al. 1990). The physiological and pathological meaning of exercise-induced protein oxidation of lungs await further study.

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