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Effect of Physical Exercise on the Content of 8-Hydroxydeoxyguanosine in Nuclear DNA Prepared from Human Lymphocytes

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Effects of exercise on the formation of 8-hydroxydeoxyguanosine (8-OH-dG), a biomarker of oxidative DNA damage, and other purine metabolites such as hypoxanthine, xanthine and uric acid were examined. Venous blood and urine were collected from swimmers and distance runners before and after the usual training. The amount of 8-OH-dG obtained from nuclear DNA of lymphocytes decreased remarkably after intermittent swimming. The amount of nuclear 8-OH-dG also declined after distance running, but this difference is statistically not significant. After each exercise, plasma concentrations of hypoxanthine, xanthine and uric acid rose significantly. Urinary excretion of hypoxanthine increased, and xanthine and uric acid decreased after exercise. The 8-OH-dG-to-creatinine ratio in urine increased slightly after swimming or running. It is supposed that the repair of oxidative DNA damage is augmented by exercise. As far as we know, this is the first report concerning the effect of exercise on oxidative damage in nuclear DNA.

Key words: Exercise — Oxidative DNA damage — 8-Hydroxydeoxyguanosine

The risk of cancer has been estimated by calculating the attributable risk in the population from various case-controlled studies. Although several epidemiological investigations indicate that increased physical activity is associated with reduced risk of cancer,[1] the results of some studies do not support these observations. Moderate treadmill exercise increased the risk of mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in rats,[2] but in the case of mice, moderate exercise and reduced energy intake decreased the incidence of DMBA-induced mammary tumors[3] and 1,2-dimethylhydrazine-induced colon tumors.[4] In rats, azaserine-induced pancreatic tumors[5] and azoxymethane-induced colon tumors[6] were reduced by voluntary exercise. In humans, a physically inactive occupation is related to increased risk of colon cancer,[7] and in women a history of active leisure is associated with a reduced risk of breast and reproductive system cancers.[8] It is conceivable that oxidative stress occurs as a result of increased metabolic rate during exercise and damages proteins, lipids and DNA *in vivo*, and thus we have examined the effect of exercise on oxidative DNA damage.

MATERIALS AND METHODS

Study population and sample collection Venous blood and urine were collected from 9 swimmers and 9 distance runners of the university sports club before and within 15 min after usual training. Mean age (year) was 20.1±1.2 (mean number±SD) in swimmers and 20.9±2.1 in runners. Mean height (cm) was 175.6±6.7 in swimmers and 172±4.3 in runners. There were significant differences between swimmers and runners in body weight (68.4±7.5 vs. 58.9±2.8 kg) and body mass index (22.2±2.2 vs. 19.9±1.4 kg/m^2). All of them were healthy non-smokers. Exercise schedule for the swimmers was interval training of over 1500 m distance in total for 90 min, and in the that of the runners was 15 km distance running in 70 min. Between swimming and running, there was a difference in relative metabolic rate (RMR) during exercise, which means the intensity of exercise as defined by the formula: RMR=(O_2 consumption during exercise−O_2 consumption at rest)/O_2 consumption at rest. Whole-body oxygen consumption during exercise increases over 10-fold in long-distance running and over 40-fold in race-speed swimming. Total energy consumption (E) is calculated by using the formula, E=(1.25+RMR)BT, B meaning the basal energy consumption per unit time at rest and T the duration of exercise. In our experiment, RMR in runners was in the range of 12–14, and the total energy consumption during 15 km running in 70 min was calculated to be 4100–4700 kJ. In the case of swimmers, energy consumption varies depending upon skillfulness and swimming style, and the oxygen consumption is high even during resting intervals to compensate the oxygen debt. Although accurate calculation of the total energy consumption was difficult in swimmers, we could roughly estimate that 2500–3100 kJ was consumed during interval training. Blood samples of 4 ml were collected in tubes containing ethylenediamine-

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tetraacetic acid. Blood cell counts were determined using a Coulter T-600 counter (Coulter Electronics, Luton, England). Plasma was removed from each sample after centrifugation and lymphocytes were collected using Ficoll-paque (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Lymphocytes were stored at −20°C until use in phosphate-buffered saline containing 10% dimethyl sulfoxide. Urine and plasma were also stored at −20°C until use.

Determination of 8-hydroxydeoxyguanosine[4] (8-OH-dG) in DNA prepared from lymphocytes The cell membrane of lymphocytes was lysed with lysis buffer containing 50 m*M* tris-HCl, 0.1 *M* NaCl, 1 m*M* diethylenetriaminepentaacetic acid (DETAPAC), 1% Triton X-100 and 10% glycerol (pH 7.5). The lysate was centrifuged at 600*g* for 5 min, and the pellet was washed three times with lysis buffer in the reaction tube (Eppendorf, Germany). Nuclear membrane of the pellet was lyzed with 30 μl of 10% sodium dodecyl sulfate and 300 μl of lysis buffer, with heating at 90°C for 3 min. Then, 20 μl of proteinase K solution (10 mg/ml, Stratagene, CA, USA) was added to digest proteins. After overnight incubation at 37°C in the dark, DNA was collected in 200 μl of 20 m*M* sodium acetate buffer (pH 4.8) using a GENECLEAN TM II (Funakoshi, Tokyo) according to the manufacturer's instructions. The DNA solution (200 μl) was denatured at 90°C for 3 min, mixed with 20 μl of nuclease P1 solution (2000 units/ml, BRL, Gaithersburg, USA) and incubated at 37°C for 30 min in the dark. Then, 20 μl of 1 *M* tris-HCl (pH 7.4) and 20 μl of *Escherichia coli* alkaline phosphatase solution (100 units/ml, Wako, Osaka) were added to the reaction tube. The mixture was incubated at 37°C for 60 min in the dark, then mixed with equal volume of HPLC buffer and finally the nucleotide solution was ultrafiltered with a Millipore filter (UFC3LGC00, Millipore, Tokyo). All enzymes used were stored in buffer containing 50% double-distilled glycerol (BRL, USA) at −20°C. Samples of 100 μl were assayed with a high-performance liquid chromatography (HPLC) apparatus connected in series with a UV detector (254 nm) and an electrochemical detector (ECD, 300 mV, oxidation), and the molar ratios of 8-OH-dG detected in ECD to deoxyguanosine detected in UV (8-OH-dG/10^5 dG) were calculated. Conditions of HPLC-UV-ECD analyses were as follows: columns, TSKgel ODS-80Tm (4.6mm×25cm×2, Tosoh, Tokyo); guard column, TSKgel ODS-80Tm (4.6mm×1.5cm, Tosoh, Tokyo); mobile phase, HPLC buffer containing 30 m*M* NaOH, 0.5 m*M* DETAPAC, 12.5 m*M* citric acid, 25 m*M* sodium acetate and 7 m*M* acetic acid (pH 5.3): methanol=92:8 (v/v); flow rate, 0.5 ml/min; temperature, 50°C. Buffers were degassed with helium during experiments. When samples of 50 μg of calf thymus DNA (Boehringer Mannheim, Germany) were assayed by this method, 8-OH-dG/10^5 dG ratios were usually in the range of 2.40–2.85. In the case of human tumor cell lines cultured in serum-free medium (ASF101, Ajinomoto, Tokyo), 8-OH-dG/10^5 dG ratios were 1.97–2.14 in PC-9 (adenocarcinoma of lung) and 6.34–6.67 in H69 (small cell carcinoma of lung).

Determination of creatinine, hypoxanthine, xanthine and uric acid in plasma and urine Plasma was diluted 10 times with the HPLC buffer, and 400 μl was ultrafiltered with a Millipore filter (UFC3LGC00, Millipore) to remove proteins, then used for HPLC assay. The plasma concentrations of creatinine, hypoxanthine and xanthine were determined by conventional HPLC-UV (254 nm) methods using 100 μl of pretreated samples. Recoveries of standards added to plasma at final concentrations of 25–100 μ*M*/liter were in the range of 102–103%, and the coefficients of variation (CV) for determinations were less than 5%. The plasma concentrations of uric acid were determined using urate C-test kit (Wako).

Urine was diluted 100 times with the same HPLC buffer and filtered through a nitrocellulose membrane (pore size=0.45 μm), and 100 μl was used for HPLC-UV (254 nm) analysis of creatinine, hypoxanthine, xanthine and uric acid. The values of CV for determinations were less than 5%.

Determination of 8-OH-dG in urine Urinary samples were pretreated as follows. Urine (2 ml) was acidified with 50 μl of acetic acid and applied to a 3-ml cation-exchange resin column (DOWEX 50W, X8) equilibrated with solution A (5% acetic acid in water:methanol=90:10). The column was washed with 2 ml of solution A, and eluted with 6 ml of solution B (50 m*M* potassium phosphate buffer (pH 7.0):methanol=90:10 (v/v)), and the eluate was applied to a 3-ml anion-exchange resin column (DOWEX 1, X2) equilibrated with solution B. The column was washed with 4 ml of solution B, then developed with 4 ml of solution C (HPLC buffer:methanol=92:8 (v/v)). The eluate was filtered through a nitrocellulose membrane (pore size=0.45 μm), and 100 μl was applied to the HPLC apparatus connected with the ECD (300 mV, oxidation, EC-8010, Tosoh). Recovery of urinary 8-OH-dG after pretreatment with the DOWEX columns was 21.9±2.9% and the CV for the signal height of 8-OH-dG peak in HPLC-ECD was 1.8% when 400 p*M* 8-OH-dG was added to urine as an internal standard.

Statistical analysis Unless otherwise stated, pair-wise *t* tests were used to evaluate the significance of differences between mean values of exercise groups, with $P<0.05$ as the criterion of significance (two-tailed *t* test).

[4] 8-Hydroxydeoxyguanosine can be named 7,8-dihydro-8-oxodeoxyguanosine.

RESULTS

Hematological examination After exercise, hematocrit (%) rose from 44.8±2.2 to 45.5±1.4 in swimmers and from 44.7±1.6 to 46±2.2 ($P<0.05$) in runners, reflecting exercise-induced hemoconcentration. Erythrocyte counts ($\times 10^4/mm^3$) also rose from 487±24 to 492±18 in swimmers and from 496±25 to 510±33 ($P<0.05$) in runners. The number of leukocytes (per mm^3) increased remarkably from 6130±1380 to 9420±2250 ($P<0.05$) in swimmers and from 5540±910 to 7730±1810 ($P<0.05$) in runners after exercise. This increase is consistent with a previous report,[9] and could not be explained by the simple hemoconcentration due to exercise.

8-OH-dG content in nuclear DNA and urine Using samples obtained before exercise, the levels of 8-OH-dG content in DNA of lymphocytes and urine were compared in each athlete. There was a correlation ($P=0.07$, simple regression F-test) between the 8-OH-dG content in DNA of lymphocytes and the level of urinary excretion of 8-OH-dG as shown in Fig. 1. This result raises the possibility that urinary 8-OH-dG could be an index of oxidative DNA damage *in vivo* if the background production of oxyradical-induced DNA damage and the repair of DNA damage were in the dynamic equilibrium state at rest. Before exercise, the levels of 8-OH-dG content in DNA of lymphocytes and urine in swimmers were higher than those of runners (in urine, $P<0.005$, unpaired two-tailed t test). The effect of exercise on 8-OH-dG content in nuclear DNA prepared from lymphocytes is shown in Table I. In spite of the anticipated oxidative stress during exercise, the 8-OH-dG/10^5 dG ratio decreased significantly after swimming. In runners, although the decrease of 8-OH-dG/10^5 dG ratio after exercise was not significant, the before-exercise level of 8-OH-dG was lower than that of swimmers ($P=0.14$, unpaired two-tailed t test).

Concentration of creatinine and purine metabolites in plasma and urine The effect of exercise on plasma concentrations of creatinine and purine metabolites is shown in Table II. Plasma concentration of creatinine, hypoxanthine, xanthine and uric acid increased significantly after swimming or running. Other investigators have likewise reported increased nucleotide metabolism after severe exercise or hypoxia.[10] The total amount of

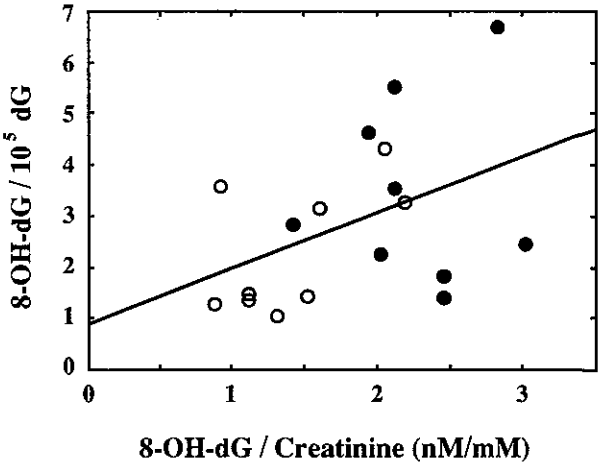

Fig. 1. Comparison of 8-OH-dG content in DNA of lymphocytes and in urine in each athlete before exercise. 8-OH-dG content in DNA of lymphocytes is expressed as the molar ratio to deoxyguanosine. Urinary 8-OH-dG is normalized to creatinine concentration, and expressed as the 8-OH-dG-to-creatinine ratio (n*M*/m*M*). Each point represents a swimmer (●) or a runner (○). The regression line for 8-OH-dG/10^5 dG versus 8-OH-dG/creatinine is shown ($r=0.431$, $P=0.07$, F-test).

Table I. Effect of Exercise on 8-OH-dG Content in Nuclear DNA Prepared from Lymphocytes

	Swimmers (n=9)	Runners (n=9)
Before exercise	3.46±1.80	2.32±1.24
After exercise	1.59±0.23[a]	2.27±1.14

The molar ratios of 8-OH-dG detected in ECD to deoxyguanosine detected in UV (8-OH-dG/10^5 dG) were calculated. Results are mean±SD.
a) $P<0.05$ compared with before exercise (two-tailed t test).

Table II. Effect of Exercise on Plasma Concentration of Creatinine and Purine Metabolites

	Swimmers (n=9)		Runners (n=9)	
	Before exercise	After exercise	Before exercise	After exercise
Creatinine (*μM*/liter)	106.1±8.8	120.2±15.9[a]	93.7±18.6	175.9±84.0[a]
Hypoxanthine (*μM*/liter)	11.61±2.91	19.09±6.35[a]	8.01±8.28	11.48±6.41[a]
Xanthine (*μM*/liter)	1.31±0.37	1.81±0.60[a]	1.39±1.25	2.74±1.47[a]
Uric acid (*μM*/liter)	405.7±44.0	433.6±36.3[a]	324.2±79.7	395.6±125.5[a]

Results are mean±SD.
a) $P<0.05$ compared with before exercise (two-tailed t test)

Table III. Effect of Exercise on Urinary Concentration of Creatinine and Purine Metabolites

	Swimmers (n=9)		Runners (n=9)	
	Before exercise	After exercise	Before exercise	After exercise
Creatinine (m*M*/liter)	15.0±6.2	14.3±3.6	18.8±5.7	29.9±6.1[a]
8-OH-dG/Cr. (n*M*/m*M*)	2.27±0.48	2.47±0.44	1.42±0.47	1.56±0.46
Hypoxanthine/Cr. (μM/m*M*)	7.57±4.03	21.55±12.26[a]	4.46±1.64	10.23±6.77[a]
Xanthine/Cr. (μM/m*M*)	7.78±3.35	4.95±2.88[a]	3.93±0.76	3.06±1.10[a]
Uric acid/Cr. (m*M*/m*M*)	0.287±0.078	0.178±0.036[a]	0.363±0.107	0.183±0.036[a]

Results are mean±SD. Concentrations of purine nucleotide metabolites are shown as molar ratio to creatinine. 8-OH-dG: 8-hydroxydeoxyguanosine. Cr.: creatinine.
a) $P<0.05$ compared with before exercise (two-tailed *t* test)

creatinine excreted daily in urine correlates with muscle mass and is believed to be fairly constant in each person, but the concentration of creatinine in urine varies depending upon daily water intake. For these reasons, we expressed the urinary concentrations of purine metabolites as the molar ratio to creatinine. In both swimmers and runners, the urinary molar ratio of hypoxanthine to creatinine increased, and those of xanthine and uric acid decreased significantly after exercise (Table III). The 8-OH-dG-to-creatinine ratio in urine increased after each exercise, but this difference is statistically not significant. In runners, urinary concentration of creatinine increased significantly after exercise, reflecting dehydration.

DISCUSSION

Reactive oxygen species such as superoxide, hydroxyl radical, hydrogen peroxide and lipid peroxide are generated in the human body under normal conditions. One of the oxidative DNA products, 8-OH-dG, is physiologically formed *in vivo*.[11, 12] Under pathological conditions, formation of 8-OH-dG is enhanced by X-ray irradiation, chemical carcinogens and other exogenous factors,[13-18] but most of it is removed by the DNA-repairing enzyme system[19, 20] and excreted in the urine.[21, 22] It is also reported that smokers have a 50% higher level of 8-OH-dG excretion than non-smokers when 24 h urine is compared,[23] and this result is consistent with our previous observation that 8-OH-dG/creatinine molar ratio in urine is 3.24 n*M*/m*M* in non-smokers (n=133) and 3.95 n*M*/m*M* in smokers (n=70) based on examination of 203 healthy males aged 22–39 (unpublished data). On the other hand, it is reported that 8-OH-dG causes miscoding of DNA both in *E. coli* and mammalian systems,[24-27] and it is supposed to trigger cancer and aging. On those theoretical grounds, the amount of 8-OH-dG excreted in the urine could be useful as an index of the risk of carcinogenesis in apparently healthy individuals.

Whole-body oxygen consumption during exercise increases over 10-fold in long-distance running and over 40-fold in race-speed swimming. During hard exercise such as race-speed swimming, blood flow is shunted away from many organs to muscles, causing hypoxia. Then, in the intervals between swimming, it is possible that the reoxygenation induces a burst of reactive oxygen species production (ischemia-reperfusion phenomenon). As reported previously, degradation of purine is accelerated by anaerobic exercise,[28] and the intensity of exercise is related to the hypoxanthine level in plasma, possibly influencing free radical formation *in vivo*.[29] Plasma hypoxanthine was elevated only after the status exceeded the anaerobic threshold (AT), and correlates with the intensity of exercise. In rats, a single bout of exhaustive swimming exercise for 10–20 min (until exhaustion) increased the xanthine oxidase (XOD) activity of lung nearly 2-fold.[30] XOD is the oxidative enzyme involved in the generation of the superoxide anion and hydrogen peroxide from hypoxanthine, producing xanthine and uric acid, which are possibly related to oxidative DNA damage, as deduced from the observation that damage to the bases in DNA is produced by the hypoxanthine/xanthine oxidase system in the presence of iron ions.[31, 32] On the other hand, cigarette smoking induces oxidative DNA damage in human peripheral leukocytes in a relatively short time.[33] For those reasons, the amount of 8-OH-dG in nuclear DNA of lymphocytes was expected to increase after anaerobic exercise. Contrary to our expectation, the 8-OH-dG level in nuclear DNA declined after intermittent swimming, and the existence of exercise-induced DNA-repairing mechanisms has to be considered.

Metabolic generation of hydrogen peroxide is proportional to dietary caloric intake, and decrease of energy consumption by dietary restriction causes a decrease of 8-OH-dG in DNA, as energy consumption is supposed to be related to free radical formation.[34] Interestingly, the intensity of exercise influences the appearance of lipid

peroxidation products: it was reported that plasma lipid peroxidation decreased in exercise at 40% of $\dot{V}O_2$max (maximum oxygen consumption), but increased at 100% $\dot{V}O_2$max.[35] Thus, the difference between swimmers and runners in the level of 8-OH-dG in nuclear DNA before exercise could be explained to some extent by the difference of intensity of daily exercise or body mass index, which reflects total caloric intake.

On the other hand, blood antioxidant systems are augmented in runners by training.[36, 37] In such cases, it is probable that the resistance to subsequent oxidative challenges such as UV, X-ray irradiation or chemical carcinogens is increased.

Although the precise mechanism by which exercise reduces 8-OH-dG in nuclear DNA prepared from lymphocyte remains unclear, it is conceivable that exercise augments the efficiency of DNA-repairing systems or the resistance of DNA to oxidative damage (exercise-induced adaptive response), resulting in a reduction of 8-OH-dG *in vivo*.

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