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Evaluation of a Multi-parameter Biomarker Set for Oxidative Damage in Man: Increased Urinary Excretion of Lipid, Protein and DNA Oxidation Products after One Hour of Exercise

HILMI ORHAN[a,b,*], BERRY VAN HOLLAND[c], BETTY KRAB[c], JANINE MOEKEN[c], NICO P.E. VERMEULEN[a], PETER HOLLANDER[c] and JOHN H.N. MEERMAN[a,d,†]

[a]*Division of Molecular Toxicology, Leiden/Amsterdam Center for Drug Research, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands;* [b]*Toxicology Department, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey;* [c]*Faculty of Human Movement Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands;* [d]*Pharmacogenomics Unit, Leiden/Amsterdam Center for Drug Research Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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The objective of the present study was to evaluate a comprehensive set of urinary biomarkers for oxidative damage to lipids, proteins and DNA, in man. Eighteen moderately trained males (mean age 24.6 ± 0.7) exercised 60 min at 70% of maximal O_2 uptake on a cycle ergometer. Urine fractions for 12 h were collected 1 day before, and for 3 consecutive days after exercise.

As biomarkers of lipid peroxidation, 8 aldehydes (i.e. propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal and malondialdehyde—MDA)and acetone were analyzed in urines by gas chromatography with electron capture detection (GC-ECD). As a biomarker of protein oxidation, *o,o′*-dityrosine was analyzed in urine samples by a recently developed isotope dilution HPLC-atmospheric pressure chemical ionization (APCI)-tandem-mass spectrometry (HPLC-APCI-MS/MS) methodology. As a biomarker of oxidative DNA damage, urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was measured by an ELISA method.

On the day of exercise, significant increases were observed in urinary excretions of acetone ($p < 0.025$, $n = 18$) and butanal ($p < 0.01$, $n = 18$) in the 12 h daytime fractions compared to the daytime fraction before exercise. The urinary acetone excretion was also significantly ($p < 0.05$) increased on the 1st day after exercise. Octanal and nonanal were increased in the daytime urine fraction on the 2nd day after exercise. However, these increases were of borderline significance ($p = 0.09$ and $p = 0.07$, respectively).

Significantly elevated urinary *o,o′*-dityrosine amounts were observed in the daytime fraction on the day of exercise ($p < 0.025$) and on the 1st day after exercise ($p = 0.07$) compared to the before exercise daytime fraction.

Excretion of urinary 8-OHdG was statistically significantly increased in the daytime fractions on the day of exercise ($p = 0.07$) and on the 1st day after exercise ($p < 0.025$) compared to before exercise daytime fraction.

Increases in urinary excretions of acetone, propanal, pentanal, MDA and 8-OHdG significantly correlated with training status (hours of exercise/week) of the volunteers, while *o,o′*-dityrosine did not.

To our knowledge, the present study is the first to evaluate a multi-parameter non-invasive biomarker set for damage to three main cellular targets of ROS. It shows that 1 h of exercise may already induce oxidative damage in moderately trained individuals and that the chosen urinary biomarkers are sensitive enough to monitor such damage.

Keywords: Exercise; Lipid peroxidation; Protein oxidation; DNA damage; Biomarker; *o,o′*-dityrosine; 8-hydroxy-2′-deoxyguanosine

Abbreviations: BMI, body mass index; DTPA, diethylenetriaminepentaacetic acid; GC-ECD, gas chromatography-electron capture detection; HPLC-APCI-MS/MS, high pressure liquid chromatography-atmospheric pressure tandem mass spectrometry; HRP, horseradish peroxidase; LPO, lipid peroxidation; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; O-PFB, O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride; RER, respiratory exchange ratio; ROS, reactive oxygen species; SEM, standard error of the mean; SRM, selected reaction monitoring; TEP, 1,1,3,3-Tetraethoxypropane; VO_2max, maximal oxygen uptake

*Corresponding author. Present address: Toxicology Department, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey Tel.: +90-312-3092958. Fax: +90-312-3114777. E-mail: hilmi@tr.net

†Tel.: +31-06-53970982. Fax: +31-71-5274277. E-mail: meerman@lacdr.leidenuniv.nl

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INTRODUCTION

Although physical exercise is highly recommended, the energy demand during physical exercise causes an increased oxygen uptake, which may increase the rate of reactive oxygen species (ROS) production. The mitochondrial electron transport chain, polymorphoneutrophil and xanthine oxidase have been identified as major sources of intracellular free radical generation during exercise.[1] Additionally, exercise may also generate free radicals by other means, including (1) increases in epinephrine and other catecholamines that may produce oxygen radicals when they are metabolically inactivated, (2) production of lactic acid that may convert a weakly damaging free radical (superoxide radical) into a strongly damaging one (hydroxyl radical), and (3) inflammatory responses to secondary muscle damage.[2–6] Therefore, the interest of the general scientific community in ROS has raised awareness of the oxygen-or exercise-paradox and has led investigators to question whether exercise stimulated "over-consumption" of oxygen might induce an oxidative stress and pose some risk to biological systems.[7] Damage to bio-molecules may occur if an increased ROS production exceeds the protective capacity of antioxidant defense mechanisms. In this context, determination of oxidative stress upon exercise by reliable and sensitive biomarkers is potentially valuable, e.g. to establish whether antioxidant supplementation might prevent deleterious effects of exercise-induced oxidative stress.

Data so far show increases in MDA in blood after an 80-km race,[8] after a 30-min treadmill test at 60 and 90% maximal oxygen uptake (VO_2max),[9] after downhill running,[10] and after incremental cycling to exhaustion in sedentary and moderately trained man.[11,12] In contrast, no increases in MDA were found after running a half-marathon,[13] after 60 min of bench-stepping exercise,[14] after maximal cycle ergometry exercise,[15] nor after incremental cycle ergometry exercise in elite athletes.[16] One hypothesis is that these equivocal results may be due to the large inter-individual variability or to the non-specificity of the analytical assays for MDA.[17] Other markers of lipid peroxidation investigated in exercise included ethane and pentane in exhalatory air in exercising healthy individuals,[18] conjugated dienes in plasma of sprint-trained athletes,[19] as well as in muscle lipids of downhill running athletes.[20] To our knowledge, urinary aldehydes were not investigated in exercise.

As to protein oxidation during/after exercise, Leeuwenburgh *et al.* reported that exercise led to a 50% increase in *o*-tyrosine, *m*-tyrosine and *o,o′*-dityrosine in the mitochondrial proteins of heart muscle of rats. There also was a transient increase in the level of *o,o′*-dityrosine in the urine of the same rats.[21] In a study on the effect of the lemon flavonoid eriocitrin on exercise-induced oxidative damage in rat liver, Minato *et al.* observed that *o,o′*-dityrosine formation increased upon exercise, while eriocitrin reversed this increase.[22]

8-OHdG is one of the best-investigated oxidative DNA damage repair products in human and animal exercise studies. Several investigators observed an increase in urinary excretion of 8-OHdG after exercise,[23] while others reported no change.[24–26] These differences may be, among others, due to the fact that some studies investigated the effects of a single bout of exercise while others investigated the effects of regular exercise.

The controversial results in literature led us to further explore oxidative stress in humans after controlled-endurance exercise in 18 volunteers using integratively and non-invasively a set of various biomarkers reflecting oxidative damage to lipids, proteins and DNA. For this purpose, we determined the urinary excretion of aldehydes (propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, MDA) and acetone as markers of lipid peroxidation, *o,o′*-dityrosine as a marker of protein oxidation, and 8-OHdG as a marker of DNA oxidation.

MATERIALS AND METHODS

Chemicals

O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (O-PFB), 3-bromofluorobenzene, acetaldehyde, propanal, butanal, pentanal, hexanal were purchased from Fluka Chemie AG (Buchs, Switzerland). Acetone, methanol, *n*-heptane, acetic acid, hydrochloric acid, ammonium acetate and sulfuric acid were obtained from Riedel-de Haen AG (Seelze, Germany). 1,1,3,3-Tetraethoxypropane (TEP), acetic acid, formic acid and sodium wolframate were purchased from Merck (Darmstadt, Germany). Sodium acetate and *n*-heptane were purchased from J.T. Baker (Deventer, The Netherlands). Sodium sulphate was obtained from Janssen Chimica (Beerse, Belgium). *p*-(ring-$^2H_4$)tyrosine (*p*-[$^2H_4$-]tyrosine) was obtained from Cambridge Isotope Laboratories (Andover, MA). 8-Hydroxy-2′-deoxyguanosine (8-OHdG), diethylenetriamine-pentacetic acid (DTPA) and horseradish peroxidase (HRP) were obtained from Sigma (St Louis, MO, USA). *p*-Tyrosine was purchased from BDH (Poole, UK). Creatinine and hydrogen peroxide were obtained from J.T. Baker (Deventer, The Netherlands). All chemicals were of the highest purity grade that was available. Nanopure water was obtained from a Milli-Q-system (Millipore, Bedford, MA, USA). MDA was obtained by hydrolysis of TEP according to the method of

TABLE I Subject characteristics of 18 male volunteers participating in the study

	Mean ± SEM
Age (year)	24.6 ± 0.7
Body weight (kg)	75.9 ± 2.1
Height (m)	1.84 ± 0.07
BMI* (kg/m^2)	22.3 ± 0.5
Training status (h sporting/week)	4.6 ± 0.4

*Body mass index.

Csallany *et al.*[27] *o,o′*-Dityrosine and *o,o′*-[$^2H_6$-]dityrosine were synthesized and purified as described previously.[28]

Subjects

Eighteen healthy males aged 19–42 volunteered to participate in this study. All subjects were moderately trained (4.6 ± 0.4 h of sporting/week) and non-smokers. A written informed consent was obtained from each person. Characteristics of the subjects are shown in Table I.

Experimental Protocol

All subjects performed the following two exercises: a VO_2max test and a one-hour exercise at 70% of VO_2max. The tests were separated by at least 1 week. Both exercises were performed on a cycle ergometer (Lode Excalibur, Groningen, The Netherlands). The one-hour exercise was performed in the morning hours.

VO_2max Test

The subjects began with a warm-up of 6 min in which they reached a target heart rate between 120 and 170 beats/min. With the heart rate and workload a prediction of the VO_2max was made using the nomogram of Åstrand.[29] A new workload was then chosen that would require an oxygen uptake of approximately 80% of the predicted VO_2max. The subjects had to cycle 3 min at this new workload. Subsequently, another prediction of the VO_2max was made, again using the nomogram ofÅstrand. A new workload was then chosen that would require an oxygen uptake of approximately 120% of the newly predicted VO_2max. The subjects had to cycle at this new workload until exhaustion.

Subjects' heart rate was monitored using a sport tester (Polar, Kempele, Finland; sampling frequency 15 s). Expired air was analyzed for volume and O_2 and CO_2 content (Oxycon-4, Mijnhardt, Bunnik, The Netherlands; sampling frequency 30 s) when the subjects cycled at a workload of 120% of the predicted VO_2max. Afterwards the subject performed a cooling-down until the subjects' heart rate was around 120 beats/min.

One-hour Exercise

Subjects began with a 5-min warm-up at 40% maximum workload before beginning the actual exercise. The actual exercise consisted of 60 min cycling at 70% VO_2max. The workload (at 70% VO_2max) was calculated using the mechanical efficiency for cycling.[29] During this exercise, heart rate (Polar sport tester, sample frequency 15 s), VO_2 (Oxycon, sample frequency 30 s) and workload were monitored. The expired air was analyzed during the last minute of every 5 min to check if the estimated workload induced the required 70% VO_2max. If this was not the case, the workload was adjusted. Tap water was provided to the subjects *ad libitum* during the warm-up and exercise. Afterwards the subject performed a cooling-down until the heart rate was around 120 beats/min.

Urine Sampling

To obtain baseline values for the urinary biomarkers investigated, subjects were asked to collect urine for 24 h on the day before and for 72 h after the one-hour exercise as 12 h fractions (daytime and nighttime). The nighttime fraction consisted of urine voided from 11 p.m. until the next morning, including the first urine void in the morning. The remaining urine was considered as daytime (Fig. 1).

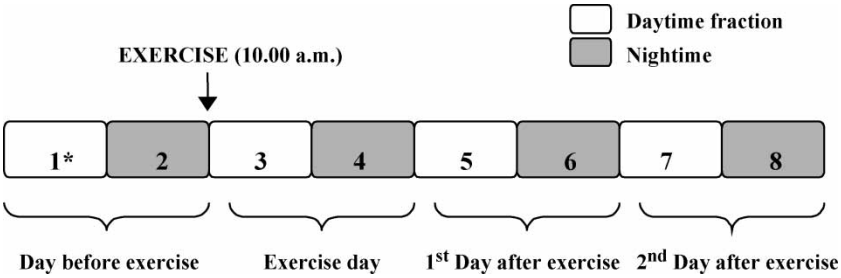

FIGURE 1 Urine collection procedure before and after the exercise. *12 h urine fraction number.

Upon arrival in the laboratory, total urine volume and pH was measured. Subsequently, 1.5 ml of each urine fraction was mixed with 0.1 ml antioxidant solution (containing 5% phenol as bactericidal agent, and 5 mM DTPA as metal chelator) and stored at −70°C until analysis.

Analysis of Aldehydes and Acetone by GC-ECD

The concentrations of 9 different LPO degradation products (i.e. propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, MDA and acetone) were determined in urine as described previously.[30] In short, urines were treated with H_2SO_4 and sodium wolframate in order to hydrolyze protein-bound aldehydes. Subsequently, O-PFB was added to the supernatant for derivatization. The oximes formed were extracted by *n*-heptane containing 3-bromofluorobenzene as external standard. After washing with HCl to remove the excess of PFB-reagent, 1 μl of heptane layer was injected into the GC column. Calibration curves for each analyte using standards in the range of 0–10 μM were made each day and data from sample analysis was considered as acceptable if the corresponding calibration curves showed a linear correlation coefficient ≥0.95. Each sample or standard was derivatized and analyzed in duplicate. Samples were analyzed in random order in different batches.

GC-ECD measurements were carried out using a Hewlett-Packard 5890 series II gas chromatograph equipped with a Hewlett-Packard 15 mCi ^{63}Ni electron-capture detector. The GC was equipped with a 30 m HP 5 (CP Sil 8 CB) column (0.32 i.d., 0.25 μm film thickness, Hewlett Packard, Amstelveen, The Netherlands). HP Chemstation software was used for data acquisition and processing. The temperature of the GC oven was programmed from 50°C (1 min) to 150°C, at 7°C/min, and then to 270°C, at 20°C/min, and kept at 270°C for 5 min. The injector and the detectors temperatures were 200 and 300°C, respectively. The helium flow rate was 2.8 ml/min.

Analysis of *o,o′*-dityrosine by LC-MS/MS

Urinary *o,o′*-dityrosine concentration was analyzed by isotope dilution HPLC-APCI-MS/MS according to Orhan *et al.*[28] Briefly, *o,o′*-[$^2H_6$]dityrosine (5 μM final concentration) was added as internal standard to the urine samples. After mixing, the samples were centrifuged at 14,000*g* for 15 min. The clear supernatant was used for the analysis. 50 μl of the clear supernatant was injected onto a Varian Chromspher 5 C18 HPLC column (25 × 4 mm) using a HPLC system consisting of a Shimadzu SCL-10 AD*vp* system controller, two LC 10 AD*vp* pumps, a CTO 10 AS*vp* column oven and an SIL 10 AD*vp* injector (Shimadzu's-Hertogenbosch, The Netherlands). A methanol gradient was applied as described.[28] The HPLC was coupled to an ion-trap mass spectrometer (Finnigan LCQ Delta, Thermoquest, Breda, The Netherlands) equipped with an atmospheric pressure chemical ionization (APCI) source. To obtain maximum selectivity, the mass spectrometer was operated in the selected reaction-monitoring (SRM) mode with positive ionization. Ionization temperature was 450°C. Other details of the instrument and assay were as described previously.[28] Samples were analyzed in random order in different batches. Calibration curves for *o,o′*-dityrosine using standards in the range of 0.05–10 μM were made freshly each day. The r^2 values ranged between 0.998 and 0.999.

8-OHdG

Urinary 8-OHdG in 10 of the volunteers before and after exercise was measured using an ELISA kit (BIOXYTECH 8-OHdG-EIA Kit. OXIS, Portland, OR, USA). Calibration, curve fitting and data analysis was done according to the instructions of the manufacturer.

Urinary Creatinine Analysis

Urinary creatinine concentrations were determined by the Jaffée method.[31]

Statistical Analysis

The data are presented as mean ± SEM (standard error of the mean). Differences in urinary excretion of biomarkers between the various urine fractions were analyzed by two-way ANOVA with the statistical software package SPSS 11.5 (SPSS Inc. Chicago, Illinois, USA) using the urine fractions as fixed factor and the individuals as random factor. When the ANOVA analyses indicated statistical differences between urine fractions, post-hoc paired *t*-tests were performed to determine significant differences between the fractions.

Associations between variables were investigated using Pearson product moment coefficient of correlation (*r*).

RESULTS

VO_2max Test and Urine Sampling

The physiological parameters of the VO_2max test and the one-hour exercise are presented in Table II. During the one-hour exercise, the subjects cycled at 68.2% VO_2max (SEM 0.68, $n = 18$), which is only slightly lower than the anticipated 70% VO_2max.

TABLE II VO_2max and one-hour exercise conditions

	Mean ± SEM	
	VO_2max test	One-hour exercise
VO_2 (l/min)	4.2 ± 0.1	2.86 ± 0.1
Heart rate (bpm)	194 ± 1.4	173 ± 2.1
Workload (W)	343 ± 8.6	208 ± 7.1
RER*	1.3 ± 0.0	0.96 ± 0.0

*RER: respiratory exchange ratio (VCO_2/VO_2).

During the second half of the one-hour exercise, the workload had to be decreased for 11 subjects (ranging from 12 to 85 Watts) to maintain 70% VO_2max. In one subject the workload had to be increased by 13 Watts.

After urine collection, urine volumes, pH and creatinine concentrations were measured. There were no significant differences in volume or creatinine concentration (Fig. 2A and B). pH values of the urines were within normal ranges (4.5–8.0) and varied for daytime fractions between 6.06 and 6.57, and for nighttime fractions between 5.78 and 6.11. The values of daytime and the nighttime fractions were not statistically significantly different.

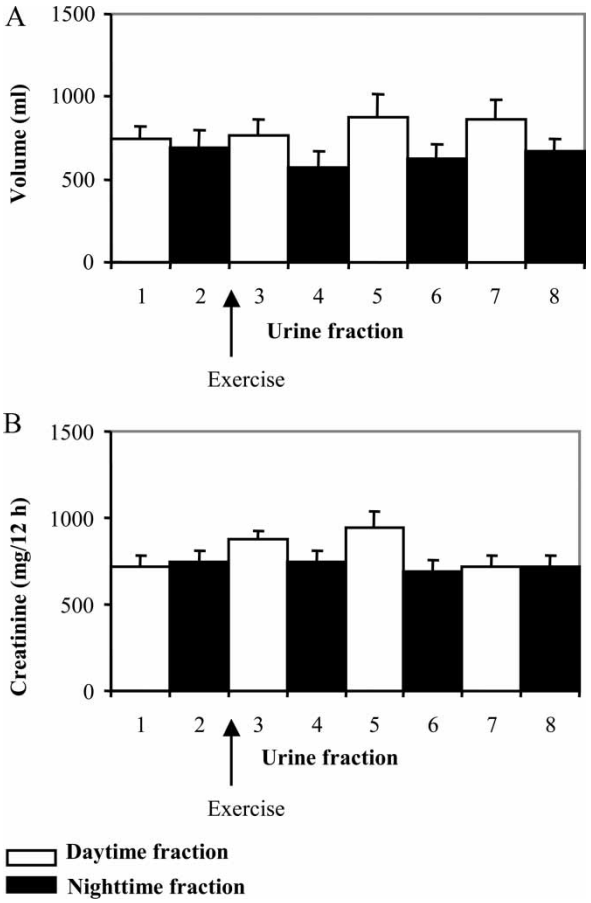

FIGURE 2 Means ± SEM of 12 h total volumes (A), and creatinine concentrations (B) of the volunteers' urines.

Aldehydes and Acetone

Formaldehyde and acetaldehyde were excluded from further analysis due to a too low reliability of the calibration curves ($r^2 < 0.95$).

The excretion of acetone, butanal, octanal and nonanal was significantly different between the various urine fractions (two-way ANOVA, $p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.025$, respectively) (Fig. 3). Post-hoc analysis indicated statistical differences between fraction 1 and 3 for acetone and butanal ($p < 0.025$, and $p < 0.01$), and between fraction 1 and 5 for acetone ($p < 0.05$) (paired t-test). The paired t-test for octanal and nonanal, however, revealed that the differences were of borderline significances ($p = 0.09$, and $p = 0.07$) (Fig. 3).

o,o′-Dityrosine

The urinary concentrations of *o,o′*-dityrosine were analyzed in all 18 volunteers. However, the data from two volunteers were excluded due to poor analytical specifications. An increase in *o,o′*-dityrosine concentrations was observed in the daytime fraction after exercise ($p < 0.025$) (Fig. 4). Also the *o,o′*-dityrosine concentration in the daytime fraction of the first day after exercise was increased, although at borderline significance ($p = 0.07$).

8-OHdG

The urinary 8-OHdG was analyzed in 10 volunteers before and after the exercise. A statistically significant difference was found between the 8-OHdG excretions in the daytime urine fraction of the first day after exercise compared to the daytime fraction before exercise ($p < 0.025$) (Fig. 5). The difference in 8-OHdG excretions between the daytime urine fraction of the day of exercise and the fraction before exercise was only of borderline significance ($p = 0.07$).

Correlations among Parameters

Correlations between training status of the volunteers and urinary excretion of the biomarkers of oxidative damage at different time points after exercise are shown in Table III. Urinary propanal and pentanal levels in the daytime fraction of the day of exercise showed significant correlations with training status of the volunteers. Also urinary levels of acetone, propanal, pentanal and MDA in the daytime fraction of the 1st day after exercise were significantly correlated with training status of the volunteers

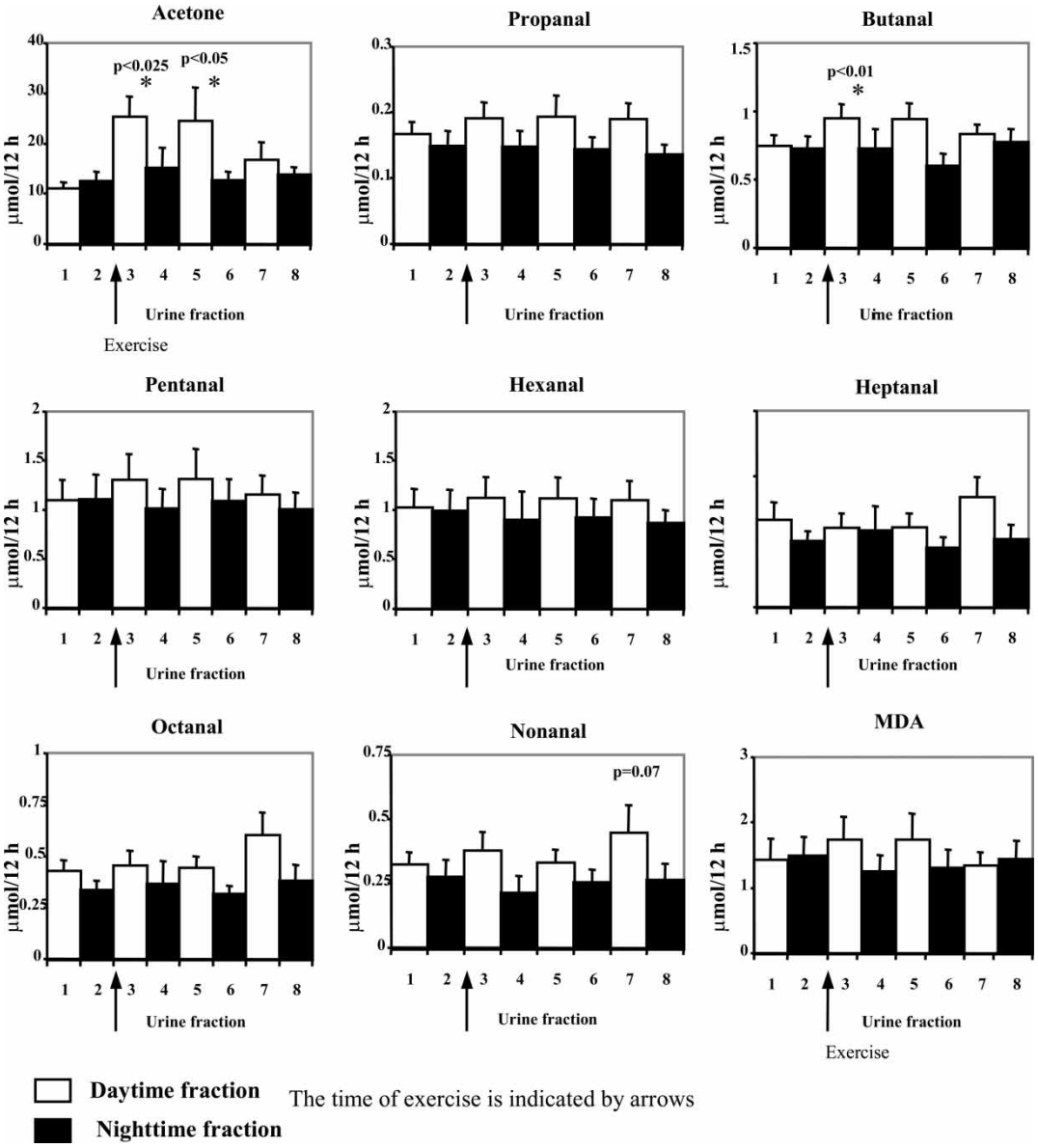

FIGURE 3 Total urinary excretions of aldehydes and acetone before and after exercise expressed as µmol per 12 h. *Statistically significantly different from the daytime fraction before exercise. Increases in octanal and nonanal excretions in daytime fractions on the 2nd day after exercise were of borderline significance. Data are presented as mean ± SEM ($n = 18$).

(Table III). Urinary 8-OHdG levels were correlated with training status of the volunteers for the nighttime fraction after exercise, and the daytime and nighttime fraction of the first day after exercise. Urinary *o,o′*-dityrosine concentration was not correlated with training status of the volunteers.

Correlations between urinary biomarkers was also observed (Table IV), i.e. between urinary excretion of *o,o′*-dityrosine on the day of exercise and the 1st day after exercise, but also with excretion of butanal on the day of exercise and between *o,o′*-dityrosine and acetone excretion on the 1st day after exercise. Excretion of 8-OHdG and acetone was also correlated on the 1st day after exercise. Furthermore, octanal and nonanal excretion was correlated on the 2nd day after exercise.

DISCUSSION

The objective of the present study was to evaluate a comprehensive set of urinary biomarkers for oxidative damage to lipids, proteins and DNA, in man. Eighteen healthy male volunteers, aged 19–42 years, were asked to cycle for 60 min at 70% of VO_2max and various biomarkers reflecting damage to lipids, proteins and DNA were analyzed in urine. To our knowledge, the present study is the first to utilize a multi-parameter non-invasive biomarker set for damage to these three main cellular targets of ROS.

Urines were collected and processed in the present study as 12 h fractions. The reason is that previously we had observed different excretion pattern of

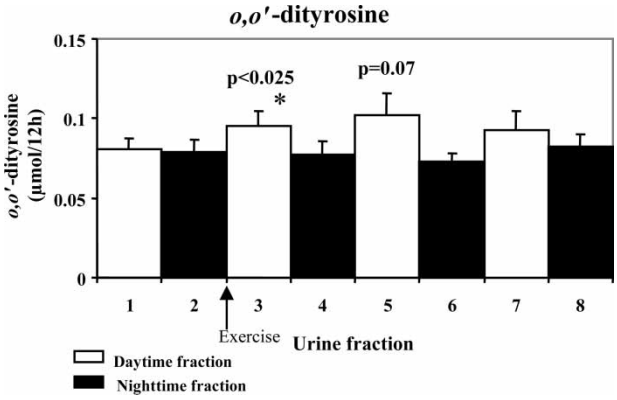

FIGURE 4 Total urinary excretion of *o,o′*-dityrosine as µmol per 12 h before and after exercise. Statistically significant increases were shown with their *p* values on top of each bar. Increase in *o,o′*-dityrosine excretion in daytime fractions on 1st day after exercise was of borderline significance. Data were presented as mean ± SEM ($n = 16$).

aldehydes[32] and of *o,o′*-dityrosine[28] during daytime and nighttime. We explained this by a different rate of basal metabolism during day and night. In the present study, higher daytime excretion was also observed for several aldehydes and *o,o′*-dityrosine but not for the other biomarkers.

So far, urinary excretion of aldehydes and acetone as biomarkers of oxidative stress has been applied only in two studies in humans. The first application was with cytotoxic drug treatment in cancer patients,[32] and the second with halothane, isoflurane and sevoflurane administration to patients.[33] These studies showed significant increases in various urinary aldehydes' excretions. The urinary background concentrations of aldehydes and acetone in controls (corrected for creatinine) between those studies and the present study are comparable.

The present study is the first application of these biomarkers to a human controlled-exercise situation. We observed statistically significant increases in urinary excretion of acetone and butanal in daytime fractions on the day of exercise, and in acetone excretion on the 2nd day of exercise. Urinary excretions of octanal and nonanal were also increased in daytime fraction on 3rd day of exercise, though the increases were of borderline significance. In previous animal experiments, we usually observed increased urinary excretion of all measured aldehydes after administration of compounds that induce clear oxidative damage and toxicity.[34–37] In those studies, acetone and butanal were among the biomarkers that showed the largest increases. Combined with the present finding that only urinary acetone and butanal excretion are increased after moderate physical exercise, it may indicate that acetone and butanal are the most sensitive biomarkers for LPO. However, formation of aldehydes and acetone may also occur by other pathways in the body. Thus, acetone formation in the body may be the result of ketosis, a process by which free fatty acids are transformed into ketone bodies.[38] The ketone bodies represent three molecules, acetoacetate, 3-β-hydroxybutyrate and acetone.

Combustion of fat molecules requires significantly more oxygen than combustion of carbohydrate molecules.[38] This probably is the reason that the VO_2max increased slightly after approximately 30 min of exercise in this study. Because the body tries to maintain blood glucose

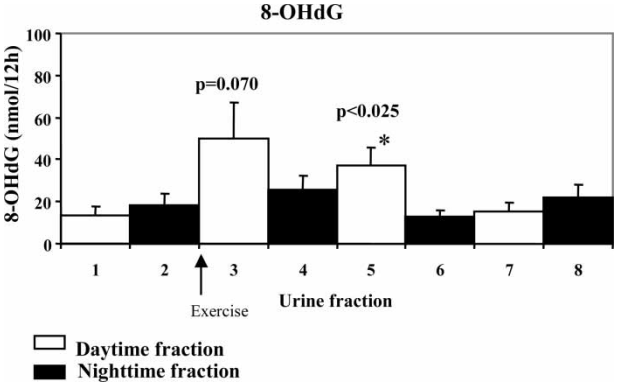

FIGURE 5 Total urinary excretion of 8-OHdG as nmol per 12 h before and after exercise. Statistically significant increases were shown with their *p* values on top of each bar. Increase in 8-OHdG excretion in daytime fractions on the day of exercise was of borderline significance. Data were presented as mean ± SEM ($n = 10$).

TABLE III Significant correlations between training status (h sporting/week) of the volunteers and urinary excretion of various biomarkers of oxidative damage after exercise

Biomarker	Fraction	Correlation (*r*)	*p*
Acetone	5	0.476	0.046
Propanal	3	0.564	0.015
	5	0.631	0.005
Pentanal	3	0.509	0.031
	5	0.491	0.039
MDA	5	0.591	0.010
8-OHdG	4	0.697	0.025
	5	0.801	0.005
	6	0.796	0.010

Values are Pearson's correlation coefficients. Statistical significances are represented by *p* value. Key for fractions: 3,4: day of exercise, 5,6: 1st day after exercise.

TABLE IV Significant correlations between urinary biomarkers that are increased after exercise

	Correlation		
Biomarkers	Fractions	(*r*)	*p*
o,o′-dityrosine-*o,o′*–dityrosine	3–5	0.678	0.003
o,o′-dityrosine–butanal	3–3	0.614	0.009
o,o′-dityrosine–acetone	5–5	0.529	0.029
8-OHdG–acetone	5–5	0.902	0.0004
Octanal–nonanal	7–7	0.743	0.0003

Values are Pearson's correlation coefficients. Statistical significances are represented by *p* value. Key for fractions: 3: day of exercise, 5: 1st day after exercise, 7: 2nd day after exercise.

levels, glucagon levels rise and insulin levels decrease. Both processes stimulate ketosis in the body. Previous research already pointed out that ketone levels rise after exercise.[39] This may (partially) explain the increase in urinary acetone excretion that we observed in this study in the urine fractions of the day of exercise and one day after exercise. However, the present study had no dietary control. Therefore, it is possible that the subjects' diet may have had some influence on acetone excretion. No such alternative routes for the formation of butanal have been described and therefore, it may represent a more reliable biomarker for oxidative damage than acetone.

Propanal, butanal, pentanal, hexanal, and MDA were already implicated as early and sensitive biomarkers for LPO in rat toxicity studies by de Zwart *et al.*[34] Propanal and hexanal are the major carbonyls formed during peroxidation of ω-3 and ω-6 fatty acids in rat liver microsomes, respectively. Some other aldehydes, such as butanal and pentanal, were also detected in such *in vitro* systems.[40,41] However, no increased urinary excretion of propanal, pentanal, hexanal and MDA was observed in this study after moderate exercise. In addition to the mentioned aldehydes, heptanal, octanal and nonanal were investigated for the first time in the present study as potential degradation products of LPO. We observed increases in urinary excretions of octanal and nonanal after exercise, though the differences were of borderline significance. There is a tendency for these longer chain aldehydes to be excreted at later time points after exercise compared to the shorter chain aldehydes such as butanal.

Urinary excretion of MDA did not increase after exercise in the present study although other products of oxidative damage were increased. There might be several possible reasons of this. First, urine is not the major excretion route for MDA. Siu and Draper[42] found that Wistar rats orally administered with radioactive [1,3-^{14}C] MDA, expired 60–70% of this MDA in the form of CO_2 within 12 h, while 5–15% was excreted through faeces and only 9–17% in the rats' urine. Marnett *et al.*[43] found similar results in Swiss mice. Moreover, MDA is also generated as a by-product of cyclooxygenase activity in platelets,[44] therefore basal levels of MDA might already be high and a relatively small increase due to exercise might not be detectable.

Amino acid residues of cellular proteins represent another critical target for the generated free radicals upon exercise. One of the most abundant amino acid is L-tyrosine and it generates long-lived tyrosyl radicals by one-electron oxidation in presence of oxidants, which may subsequently react with another L-tyrosine molecule and form *o,o′*-dityrosine.[45] Urinary *o,o′*-dityrosine concentration in humans was reported in two studies previously.[28,46] The first study reported urinary *o,o′*-dityrosine concentration in healthy humans in the range of 3.1–25.2 μmol/mol creatinine. The mean urinary *o,o′*-dityrosine concentration in the second study was 29.4 μmol/mol creatinine. In the present study, urinary *o,o′*-dityrosine concentrations before exercise range from 7.4 to 33.5 μmol/mol creatinine. Thus, urinary *o,o′*-dityrosine concentrations are very comparable in all three studies.

There is only one other human study concerning analysis of *o,o′*-dityrosine in exercise.[47] These authors reported that the ratio of fluorescence due to *o,o′*-dityrosine to that due to tryptophan in plasma proteins increased after exercise.[47] In a rat study, Leeuwenburgh *et al.* showed that exercise led to a 50% increase in *o,o′*-dityrosine in the mitochondrial proteins of heart muscle. There was also a transient increase in the level of *o,o′*-dityrosine in the urine of exercised rats.[48] Our present finding that *o,o′*-dityrosine urinary excretion increased upon exercise is in accordance with those data and suggests that urinary *o,o′*-dityrosine may be a reliable, sensitive and early biomarker of protein oxidation.

An interesting recent observation is the formation of aldehydes from amino acids in the body. Under conditions of inflammation, neutrophils employ the myeloperoxidase–H_2O_2–Cl^- system to oxidize common α-amino acids to yield a family of reactive aldehydes.[49] Oxidation of Val, Leu, and Asp residues by $HO^{\cdot}/O_2$ was shown to result in the release of a family of carbonyls (including formaldehyde, acetone, isobutyraldehyde, and glyoxylic acid) via β-scission of alkoxyl radicals.[50] The aldehydes formed from aromatic amino acids such as L-tyrosine and phenylalanine are *p*-hydroxyphenylacetaldehyde and phenylacetaldehyde, respectively.[49,51] The importance of these aldehydes as urinary biomarkers require further studies and offer an interesting area of research.

8-OHdG is usually believed to be a good biomarker of DNA oxidation.[52] Urinary 8-OHdG levels in healthy control subjects have been reported in several studies.[25,53–55] By using the reference value for creatinine (1.3 g/24 h) and an average body weight (70 kg),[56] these values correspond to 0.94–2.4 μmol/mol creatinine. We determined the urinary 8-OHdG concentration range before exercise as 0.7–4.2 μmol/mol creatinine in the present study.

Thus, our urinary levels are very comparable with the previously reported concentrations. However, there remain concerns about the specificity of the ELISA assay for 8-OHdG used in this study. Although a reasonably good correlation ($r = 0.88$) was obtained between urinary 8-OHdG concentrations measured by ELISA and HPLC-ECD in one study,[57] in another study a good correlation ($r = 0.833$) was obtained only after pre-purification

of urine samples by HPLC before the ELISA.[58] The conclusion from that study was that the ELISA could be applied to studies comparing relative urinary 8-OHdG values (as is done in our present study), if the studies do not require determination of the exact concentration of 8-OHdG in urine. It has been suggested that there are three principal sources of 8-OHdG: DNA repair, the diet, and cell turnover. In the present study, the diet of the subjects was not controlled with the exception that they were non-smoker individuals. It is not expected that cell turnover is increased after exercise under the present study conditions, since cycling is not known to cause cell damage. It has very recently been reported that the two DNA repair enzymes, hOGG1 and MTH1, were slightly up regulated upon marathon exercise. This observation led the idea that this up-regulation might be the result of an exercise induced adaptation process.[59] Whether similar inducible repair mechanisms exist for oxidative protein damage remains to be established although increased proteasome activity after exercise has been reported.[60] This may, however, depend on the type of exercise and time after exercise.[61,62]

The level of urinary excretion of several biomarkers in the present study correlated with training status (hours of sporting/week) of the volunteers reasonably well. The correlations with training status may partially be explained by increased oxygen uptake in well-trained individuals because there were also correlations between urinary excretions of these compounds and VO_2max, although these correlations were not as strong as with training status. However, training status was an independent factor not correlated with body weight or height. This may suggest that the higher excretion of biomarkers in well-trained individuals might be the result of induced, higher levels of repair systems leading to a more rapid repair and, consequently, higher levels of biomarkers in urine in the first days after exercise. The correlations between various urinary biomarkers in urinary fractions 3, 5 and 7 support the conclusion that there is simultaneous oxidative damage to lipids, proteins and DNA after one hour of exercise.

Because our present study is "self-controlled", it seems necessary to confirm our present findings in a randomized and controlled study with a larger number of volunteers. It may also be interesting to study the influence of different levels of training on the excretion of biomarkers.

CONCLUSION

The present study shows that 60 min cycling at 70% of maximal O_2 uptake may induce oxidative stress in moderately trained individuals and that the urinary biomarkers, chosen to reflect lipid, protein and DNA damage, are sensitive enough to monitor such stress. The present study is the first application of such a large multiple biomarker sets of LPO, DNA and protein oxidation upon human exercise. They seem promising tools for further studies and they might be useful for sports-physiological studies under more extreme conditions, e.g. to study the effectiveness of antioxidant supplementation during exercise.

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