Urinary excretion of cytokines versus their plasma levels after endurance exercise

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ABSTRACT

It has been consistently shown that circulating levels of interleukin (IL)-6, IL-8, IL-1 receptor antagonist (IL-1ra) and IL-10 increase remarkably following endurance exercise longer than 2 h such as marathon and triathlon races. However, no studies have compared changes in the plasma and urinary levels of these cytokines after endurance exercise, including the recovery period. In the present study, we investigated kinetic changes in the urinary excretion of cytokines following endurance exercise up to 3 h after exercise to evaluate the magnitude of change in comparison to the plasma levels and to explore the possible biological significance and the mechanisms of cytokine dynamics following exercise. Fourteen male athletes participated in a duathlon race consisting of 5 km of running, 40 km of cycling, and 5 km of running. Venous blood and urine samples were collected before, immediately after, 1.5 h and 3 h after the race. Plasma and urine were analyzed using enzyme-linked immunosorbent assays (ELISA). Plasma concentrations of IL-1β, IL-1ra, IL-6, IL-8, IL-10 and monocyte chemotactic protein (MCP)-1 increased significantly after the race, whereas tumour necrosis factor (TNF)-α, IL-2, IL-4, IL-12 and interferon (IFN)-γ did not change significantly. Urinary concentrations of IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ and MCP-1 increased significantly after the race. When the urine concentrations were adjusted by creatinine concentration, urine volume and sampling time, the increases of IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 were evident and these were notably present in urine of the stressed athletes suffering from renal tubular epithelial damage. The present study provides new evidence that the kinetics and magnitude of changes in urinary cytokine concentrations differ from plasma cytokine concentrations following endurance exercise, especially, in the recovery

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period several hours after exercise, and that the damaged kidney might be responsible at least in part for the kinetics of some cytokines. Urinary cytokines may be sensitive biomarkers of the impact of exhaustive exercise workload on renal damage and inflammation in the recovery period after endurance exercise.

Key words: exertion, anti-inflammation, chemokine, urinalysis, acute kidney injury (AKI)

INTRODUCTION

Cytokines are potent intercellular signalling molecules that usually act within the local tissues in an autocrine or paracrine manner. However, systemic spillover of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 from damaged tissues can occur in response to a variety of serious insults such as severe trauma, burns, hemorrhagic shock, sepsis and ischaemia-reperfusion injuries, which are integrated as a systemic inflammatory response syndrome (SIRS) and multiple organ failure syndrome characterized by hypercytokinaemia (1, 19, 46). For example, in a sepsis model, TNF-α is the first cytokine released systemically, and peaks within 2 h after the onset of sepsis, followed shortly thereafter by peaks of IL-1, and then IL-6 within 4 h after the disease onset (1, 37). These pro-inflammatory cytokines induce pyrogenesis and promote subsequent acute inflammatory responses such as neutrophilia, lymphocytopenia by inducing IL-8, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 (19, 37). In contrast to these pro-inflammatory cytokines, anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra), IL-4 and IL-10 may prevent inflammatory tissue damage but cause immunosuppressive states of the body (10, 16, 46, 52).

The plasma concentrations of the classical pro-inflammatory cytokines, TNF-α and IL-1β, in general, do not increase immediately after exercise (4, 9, 14, 24, 33-35, 45-49). In contrast, IL-6 is the first cytokine present in the circulation during exercise (9, 14, 17, 24-26, 28, 37, 39, 46). The level of circulating IL-6 increases up to 100-fold, depending on the intensity and duration of endurance exercise, and declines in the post-exercise period (28-30, 32-35, 45-49). Contracting skeletal muscle is the main source of IL-6 in the circulation. In response to exercise, an increase in the IL-6 mRNA content in the contracting skeletal muscle is detectable after 30 min of exercise, and up to 100-fold increases of the IL-6 mRNA content occur at the end of the exercise bout (17, 25, 33-35). In addition, it is demonstrated that both intramuscular IL-6 mRNA expression and protein release are enhanced when intramuscular glycogen is low, indicating that IL-6 is involved in energy metabolism during endurance exercise (17, 33-35, 48).

Concerning immunomodulatory cytokines that are potent functional activators of cellular immunity (29, 30, 36, 37, 38), there are no reports showing significant increases in plasma IFN-γ concentration after exercise, whereas there are several reports demonstrating a decrease in the plasma concentration of IL-2 after exercise (11, 28-30, 39, 53). Interestingly, many studies have shown that the capacity of blood leucocytes to produce IL-2 and IFN-γ decreases after endurance exercise (28-30, 39, 53). The findings that pro-inflammatory and immunomodulatory

cytokines only increase to a small extent, or are even down-regulated after exercise, could well be attributed to the actions of anti-inflammatory cytokines such as IL-1ra, IL-4 and IL-10. Indeed, we previously observed more than 200-fold increase of plasma IL-1ra after a marathon race (45) and delayed secretion of IL-4 several hours after short-duration maximal-intensity exercise (46). Furthermore, we also observed renal excretion of several cytokines, which may be one of the underlying mechanisms that plasma cytokines either remain unchanged or exhibit relatively small, delayed increments following exhaustive exercise.

Although many researchers have consistently shown that circulating levels of IL-6, IL-8, IL-1ra and IL-10 increase remarkably following endurance exercise longer than 2 h such as marathon and triathlon races (4, 14, 24, 33-37, 45-49), less is known concerning changes in urinary cytokine excretion after endurance exercise and during the recovery period (29, 30, 46, 47, 53). The present study investigated kinetic changes in the urinary levels of cytokines following endurance exercise lasting several hours to evaluate the magnitude of change in comparison to the plasma concentrations of the same cytokines. It is known that deterioration of glomerular filtration rate and oliguria are induced following endurance exercise (50), and that endurance exercise causes rhabdomyolysis (5, 31, 41), produces reactive oxygen species (ROS), and contributes to the onset of acute renal failure through reduced renal blood flow and damage of tubular cells (15). In the previous studies, after renal ischaemia-reperfusion, IL-2, IL-8, IL-10, MCP-1 and IFN-γ appeared in the kidney (7, 8, 12, 21, 42, 51). Accordingly, in this study, we evaluate acute kidney injury (AKI) following endurance exercise in relations with the changes of plasma and urine levels of various cytokines. Our hypothesis was that plasma levels of cytokines depend on urinary excretion and the excreted cytokines into urine might provide important information on renal damage and inflammation after endurance exercise.

METHODS

Subjects

Fourteen male triathletes (age 28.7 ± 7.9 (mean ± SD) yr and body mass 63.2 ± 6.0 kg) participating in the 19th Kikunotsuyu duathlon race, held on March 16th 2008, volunteered for this study. The protocol was approved in advance by the institutional ethics committee of Waseda University. None of the athletes had been ill in the month prior to the race, which was confirmed from medical questionnaire.

Race conditions

All participants agreed to avoid the use of vitamin/mineral supplements, herbs and medications from the day prior to the race until the end of experimental period (3 h post-exercise). They ate the same breakfast containing 574 kcal with 22.1 g protein, 13.7 g fat, and 88.8 g carbohydrate at 08:30. They emptied their bladders before 08:30 and just before the race to eliminate any effect of remaining urine. The race consisted of 5 km of running, 40 km of cycling, and 5 km of running, and began at 14:00. The weather was fair, with the ambient temperature at 24.6 degrees Celsius (°C). They did not exercise for approximately 18 h before

the pre-race blood and urine sampling. With the subjects resting quietly, the pre-race blood and urine samples (Pre) were collected at 10:30. They ate the same lunch containing 211 kcal with 9.3 g protein, 2.4 g fat, and 38.6 g carbohydrate at 11:00. The post-race blood and urine samples were collected immediately (0 h), 1.5 h (1.5 h) and 3 h (3 h) after the race. All participants drank the same quantity of fluid during exercise. They each drank 600 ml of fluid before the race, 1400 ml of fluid during the race and 1500 ml of fluid until 3 h after the race, respectively.

Blood and urine sampling

Approximately 7 ml of blood was drawn by a standard venipuncture technique from the antecubital vein using vacutainers containing no additive or disodium EDTA as an anticoagulant to obtain serum and plasma samples, respectively. Collected blood samples containing no additives were allowed to clot at room temperature for one hour before centrifugation at 1000 *g* for 10 min for serum preparation, whereas blood samples containing disodium EDTA were centrifuged immediately for plasma preparation. Plasma was stored at –80 °C until the day of analysis. Serum concentrations of creatinine (Cr) and albumin (ALB) were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo), and urine osmolality was determined using an auto-osmometer (OSMOSTAT, Kyoto Daiichi Kagaku, Kyoto).

Urine samples were collected directly in graduated cylinders and the volume was measured and then approximately 8 ml of urine was stored at 4 °C without centrifuge until analysis of sediments, respectively. Remaining urine samples were centrifuged immediately at 1000 *g* for 10 min to remove sediments, and the supernatants were stored at –80 °C until the day of analysis. Urinary concentrations of Cr, ALB and *β-N*-acetyl-D-glucosaminidase (NAG) activity were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo). White blood cells and red blood cells in urinary sediments were counted by flow cytometer (UF-1000i, Sysmex, Kobe), and other cells and casts in urinary sediments were analyzed by microscope (x400 high power field: HPF).

Assays for cytokines

Cytokine concentrations were measured in EDTA-plasma and urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. Concentrations of IL-1β, IL-6, IL-12 and TNF-α were measured with Quantikine high sensitivity (HS) kits (R&D Systems, Inc., Minneapolis, MN). IL-1ra and MCP-1 were measured with Quantikine kits (R&D Systems, Inc.). IL-2, IL-4, IL-8, IL-10 and IFN-γ were measured with OptEIA kits (Beckton Dickinson Biosciences, San Diego, CA). For all assays, the absorbance was measured spectrophotometrically on a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA), and the concentration of each cytokine was calculated by comparison with a calculation curve established in the same measurement.

Data analyses

Data are presented as means ± SD. Statistical validation was made using Friedman's test. If significance was detected, the Scheffe method was used for multiple comparisons. Statistical significance was evaluated at $p < 0.05$.

RESULTS

Plasma and urine cytokines

As shown in Table 1, the plasma concentrations of TNF-α did not change significantly following the race. Plasma IL-1β concentration increased significantly at 1.5 h after the race (16-fold) and remained elevated at 3 h after the race (5-fold) compared with the pre-exercise values. Plasma IL-1ra concentration showed more substantial changes significantly above pre-exercise values at immediately after the race (100-fold) and at 1.5 h post-exercise (169-fold), and at 3 h post-exercise (66-fold). The plasma concentrations of IL-6, IL-8, IL-10 and MCP-1 also increased significantly at immediately after the race, but decreased thereafter. The plasma concentrations of IL-2, IL-4, IL-12 and IFN-γ did not change significantly following exercise.

Urine volume and urinary creatinine concentration changed markedly following exercise as shown in the bottom of Table 1. Therefore, the urinary concentrations of cytokines are reported as raw data, those corrected for creatinine concentration, the gross amount (raw concentration × urine volume), and the gross amount per minute (urinary excretion rate). Urinary TNF-α did not increase significantly. Urinary IL-1ra, IL-6, IL-12 and MCP-1 were significantly higher after the race compared with pre-race values, but these differences were no longer significant when corrected for creatinine. Urinary levels of IL-2 (59-fold), IL-4 (348-fold), IL-8 (20-fold), IL-10 (54-fold) and IFN-γ (18-fold) were significantly elevated at 3 h post-exercise compared with pre-exercise values.

Urinary sediments

As shown in Table 2, renal tubular epithelial cells and renal tubular epithelial casts were observed in the urinary sediments of 7 subjects, among the fastest 8 subjects for the race time. Granular casts were also observed in the urinary sediments of 6 of these 7 subjects. White blood cells were more dominant than red blood cells in most subjects, and 13 of the 14 subjects (1~3 counts/ every field) recovered at 3 hours after the race, but only one subject (No.6) did not recover (11~15 counts/ every field). Based on these results, we analyzed between two subgroups that were divided according to existence (damaged group, n=7) or non-existence (minor-damaged group, n=7) of renal tubular epithelial cells in the urinary sediments as follows.

Parameters of renal functions

As shown in Figure 1, in the damaged group, serum Cr concentration increased significantly at 0 h post-exercise (0.54mg/dL; 1.66-fold) and at 1.5 h after the race (0.36mg/dL; 1.44-fold) compared with the pre-exercise values, whereas serum Cr concentration increased significantly at 0 h post-exercise (0.32mg/dL; 1.41-fold) as compared to pre-exercise values in the minor-damaged group. In the minor-damaged group, urinary excretion volume per minute (excretion volume rate) exhibited no significant change. Excretion volume rate in the damaged group and creatinine clearance (Ccr) in both groups decreased after the race compared with the pre-exercise values. Ccr and excretion volume rate in both groups did increase from 0 h, but still remained below the pre-exercise levels at 3 h after exercise (damaged group only: Ccr $p<0.05$). There was a significant decrease in

Table 1. Changes in plasma and urinary cytokines following the duathlon race.

	Unit	Pre	0 h	1.5 h	3 h	Fried-man test	Scheffe test
TNF-α-P	pg/mL	0.31 ± 0.45	0.10 ± 0.04	0.26 ± 0.28	0.92 ± 1.69	-	-
TNF-α-U	pg/mL	2.1 ± 2.1	2.6 ± 2.0	4.2 ± 6.0	2.3 ± 2.9	-	-
	pg/mgCr	1.8 ± 1.6	2.0 ± 1.8	1.8 ± 2.0	2.8 ± 3.8	-	-
	pg	442.4 ± 450.7	227.0 ± 250.9	251.9 ± 302.8	404.5 ± 580.2	-	-
	pg/min	3.7 ± 3.8	1.3 ±1.3	2.8 ± 3.4	4.5 ± 6.5	-	-
IL-1ß-P	pg/mL	0.06 ± 0.12	0.43 ± 1.21	0.96 ± 2.03	0.29 ± 0.38	**	Pre-1.5 h* Pre-3 h**
IL-1ß- U	pg/mL	0.97 ± 0.74	6.32 ± 9.43	10.82 ± 27.73	1.66 ± 2.76	**	0 h-3 h*
	pg/mgCr	0.90 ± 0.71	4.53 ± 5.88	4.15 ± 8.42	1.78 ± 2.84	*	0 h-3 h*
	pg	199.9 ± 180.9	267.0 ± 283.9	480.6 ± 772.7	269.2 ± 468.5	-	-
	pg/min	1.7 ± 1.5	2.2 ± 2.5	5.3 ± 8.6	3.0 ± 5.2	-	-
IL-1ra-P	pg/mL	50.5 ± 47.0	5058.7 ± 8270.7	8223.7 ± 8692.5	3333.9 ± 4540.9	**	Pre-0 h** Pre-1.5 h** Pre-3 h*
IL-1ra-U	pg/mL	189.8 ± 222.8	1658.2 ± 2313.5	7751.2 ± 13390.6	2990.1 ± 8537.6	*	Pre-1.5 h*
	pg/mgCr	160.9 ± 186.1	1182.5 ± 2507.6	3175.5 ± 6314.9	1991.1 ± 4471.9	-	-
	pg	28828 ± 24904	107482 ± 236347	476039 ± 1105060	282657 ± 597769	-	-
	pg/min	240.2 ± 207.5	865.9 ± 1992.0	5289.3 ± 12278.4	3140.6 ± 6641.9	-	-
IL-2-P	pg/mL	0.14 ± 0.11	0.26 ± 0.29	0.28 ± 0.35	0.23 ± 0.31	-	-
IL-2-U	pg/mL	6.3 ± 13.5	21.7 ± 48.2	305.3 ± 383.1	373.8 ± 382.1	*	-
	pg/mgCr	4.3 ± 7.0	7.2 ± 10.0	117.2 ± 165.5	195.6 ± 197.4	**	Pre-3 h** 0 h-3 h*
	pg	947.6 ± 1369.8	1005.0 ± 1410.9	18316.2 ± 26638.7	25476.4 ± 28349.0	*	0 h-3 h*
	pg/min	7.9 ± 11.4	5.1 ± 5.8	203.5 ± 296.0	283.1 ± 315.0	**	Pre-3 h* 0 h-3 h*
IL-4-P	pg/mL	0.25 ± 0.05	0.36 ± 0.14	0.30 ± 0.10	0.25 ± 0.08	-	-
IL-4-U	pg/mL	118.0 ± 403.2	1260.6 ± 2890.1	34928.9 ± 61276.3	41073.7 ± 61409.3	*	Pre-3 h*
	pg/mgCr	109.3 ± 384.3	329.1 ± 603.4	10529.2 ± 18532.6	20119.0 ± 30392.5	**	Pre-3 h* 0 h-3 h*
	pg	29472 ± 104996	35335 ± 64621	1786140 ± 3634239	2272571 ± 3488557	**	Pre-3 h* 0 h-3 h**
	pg/min	245.6 ± 875.0	262.2 ± 454.5	19846.0 ± 40380.4	25250.8 ± 38761.7	**	Pre-3 h* 0 h-3 h**

IL-6-P	pg/mL	0.41 ± 0.40	11.84 ± 8.43	4.42 ± 3.67	1.61 ± 1.52	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
IL-6-U	pg/mL	0.20 ± 0.15	0.36 ± 0.29	0.58 ± 0.45	1.58 ± 3.38	**	Pre-0 h* Pre-1.5 h**
	pg/mgCr	0.17 ± 0.07	0.23 ± 0.16	0.28 ± 0.20	0.79 ± 1.59	-	-
	pg	39.9 ± 19.3	22.2 ± 18.0	41.2 ± 31.5	75.3 ± 95.5	**	Pre-0 h* 0 h-3 h**
	pg/min	0.33 ± 0.16	0.14 ± 0.09	0.46 ± 0.35	0.84 ± 1.06	**	Pre-0 h* 0 h-1.5 h** 0 h-3 h**
IL-8-P	pg/mL	16.6 ± 10.4	44.9 ± 20.2	32.8 ± 12.6	22.1 ± 11.2	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
IL-8-U	pg/mL	9.0 ± 8.9	31.8 ± 28.7	153.2 ± 158.4	184.6 ± 145.2	**	Pre-1.5 h** Pre-3 h*
	pg/mgCr	7.7 ± 6.6	18.8 ± 15.2	60.9 ± 70.5	113.2 ± 70.8	**	Pre-3 h** 0 h-3 h*
	pg	1698 ± 1658	1649 ± 1364	9325 ± 11202	15150 ± 10602	**	Pre-3 h** 0 h-3 h**
	pg/min	14.2 ± 13.8	11.7 ± 11.1	103.6 ± 124.5	168.3 ± 117.8	**	Pre-3 h** 0 h-1.5 h* 0 h-3 h**
IL-10-P	pg/mL	3.1 ± 8.2	16.1 ± 31.4	11.5 ± 38.4	10.7 ± 37.0	**	Pre-0 h** 0 h-3 h**
IL-10-U	pg/mL	6.7 ± 8.5	34.1 ± 48.0	311.7 ± 376.5	364.2 ± 337.9	**	Pre-3 h*
	pg/mgCr	5.7 ± 7.5	14.1 ± 20.4	115.6 ± 147.3	203.3 ± 170.4	**	Pre-3 h** 0 h-3 h**
	pg	1352 ± 1959	1808 ± 2414	18318 ± 25117	26594 ± 23686	**	Pre-3 h** 0 h-3 h**
	pg/min	11.3 ± 16.3	13.7 ± 20.2	203.5 ± 279.1	295.5 ± 263.2	**	Pre-3 h** 0 h-3 h**
IL-12-P	pg/mL	0.20 ± 0.08	0.30 ± 0.17	0.25 ± 0.13	0.21 ± 0.08	-	-
IL-12-U	pg/mL	1.5 ± 1.1	4.4 ± 4.3	3.0 ± 3.0	1.4 ± 1.1	**	Pre-0 h* 0 h-3 h*
	pg/mgCr	1.4 ± 1.3	2.9 ± 3.4	1.4 ± 1.3	2.3 ± 3.7	-	-
	pg	298.1 ± 257.1	182.9 ± 112.9	198.9 ± 188.2	303.5 ± 429.1	-	-
	pg/min	2.5 ± 2.1	1.4 ± 1.0	2.2 ± 2.1	3.4 ± 4.8	-	-

IFN-γ-P	pg/mL	0.04 ± 0.71	0.15 ± 0.29	0.07 ± 0.08	0.08 ± 0.11	-	-
IFN-γ-U	pg/mL	11.8 ± 24.0	45.7 ± 78.2	179.0 ± 189.5	212.6 ± 165.4	**	Pre-1.5 h** Pre-3 h* 0 h-1.5 h*
	pg/mgCr	8.4 ± 12.1	17.0 ± 23.1	73.6 ± 85.4	129.5 ± 86.5	**	Pre-1.5h* Pre-3 h** 0 h-3 h**
	pg	1828 ± 2397	2125 ± 2590	11201 ± 13173	17481 ± 13265	**	Pre-3 h* 0 h-1.5 h** 0 h-3 h**
	pg/min	15.2 ± 20.0	12.0 ± 13.5	124.5 ± 146.4	194.2 ± 147.4	**	Pre-3 h* 0 h-1.5 h** 0 h-3 h**
MCP-1-P	pg/mL	2.8 ± 2.6	7.9 ± 3.5	6.3 ± 2.9	4.0 ± 2 .5	**	Pre-0 h** Pre-1.5 h** 0 h-3 h**
MCP-1-U	pg/mL	1.3 ± 0.9	8.7 ± 24.8	5.1 ± 8.4	8.4 ± 16.3	**	Pre-1.5 h*
	pg/mgCr	1.1 ± 0.7	2.6 ± 4.7	1.7 ± 1.3	4.5 ± 6.8	-	-
	pg	260.9 ± 179.1	206.3 ± 329.6	240.4 ± 157.4	661.5 ± 1064.4	**	0 h-3 h*
	pg/min	2.2 ± 1.5	1.5 ± 2.4	2.7 ± 1.8	7.4 ± 11.8	**	0 h-3 h*
Urine volume	mL	220.7 ± 107.2	57.5 ± 46.4	83.1 ± 44.1	191.7 ± 201.3	**	Pre-0 h**
Cr-U	g/L	1.1 ± 0.3	2.0 ± 1.4	2.4 ± 1.5	1.3 ± 0.8	**	Pre-1.5 h*
Osmotic Pressure-S	mOsm/L	296.1 ± 5.1	299.8 ± 8.1	288.6 ± 5.3	288.3 ± 5.4	**	Pre-1.5 h* Pre-3 h* 0 h-1.5 h** 0 h-3 h**
Osmotic Pressure-U	mOsm/L	719.6 ± 193.4	482.4 ± 258.7	630.6 ± 225.5	527.9 ± 337.0	*	Pre-0 h*

Values: means ± SD (n=14). Statistics: $^{**}p < 0.01$, $^{*}p < 0.05$.
-P: plasma. -U: urine. Cr: creatinine was used for the adjustment of urine concentration. The gross amount of urine (pg) was calculated by the raw concentration (pg/mL)×urine volume (mL). Urinary excretion rate was calculated by the raw concentration (pg/mL)×urine volume (mL) / one minute (min). Abbreviations: interleukin (IL)-1 receptor antagonist (IL-1ra), tumour necrosis factor (TNF), interferon (IFN), monocyte chemotactic protein (MCP).

Ccr at 0 h following exercise in the damaged group ($p<0.05$). Urinary excretion rate of ALB increased significantly at 0 h (30.3-fold) after the race in the minor-damaged group, and until 1.5 h (43.4-fold) after exercise in the damaged group, but were then reduced. The change in urinary excretion rate of NAG activity in the minor-damaged group was not significant. In the damaged group, urinary excretion rate of NAG activity was not significantly different from pre-exercise at 0 h, however, levels then increased significantly compared with 0 h post-exercise. Urinary excretion rate of NAG activity did not return to pre-exercise levels at 3 h

Table 2. Cells and casts in the urinary sediments following the duathlon race.

A: renal tubular epithelial cell

race time (min)	subject No.	Pre	0 h	1.5 h	3 h
99.6	1		+	+	
101.1	2			+	+
103.0	3		+		
108.7	4				
109.4	5			+	
110.0	6			+	+
111.8	7		+		
113.0	8			+	
118.2	9				
124.1	10				
124.8	11				
138.7	12				
152.3	13				
167.4	14				

B: renal tubular epithelial cast

Pre	0 h	1.5 h	3 h
	+	+	
		+	+
	+		
		+	
		+	+
	+		
		+	

C: granular cast

Pre	0 h	1.5 h	3 h
	1-3/s.f.	1-3/e.f.	
		1-3/e.f.	1-3/s.f.
	1-3/s.f.		
		1-3/s.f.	
		1-3/s.f.	
		1-3/e.f.	
	1-3/s.f.		

D: white blood cell

subject No.	Pre	0 h	1.5 h	3 h
1	1-3/s.f.	1-3/e.f.	7-10/e.f.	1-3/e.f.
2	1-3/s.f.		7-10/e.f.	1-3/e.f.
3	1-3/s.f.	7-10/e.f.	4-6/e.f.	1-3/e.f.
4	1-3/s.f.	7-10/e.f.	1-3/s.f.	1-3/s.f.
5	1-3/e.f.	11-15/e.f.	16-20/e.f.	1-3/e.f.
6	1-3/s.f.	1-3/e.f.	11-15/e.f.	11-15/e.f.
7	1-3/s.f.	7-10/e.f.	1-3/e.f.	1-3/s.f.
8	1-3/s.f.		7-10/e.f.	1-3/e.f.
9	1-3/s.f.	16-20/e.f.	7-10/e.f.	1-3/e.f.
10	1-3/s.f.	16-20/e.f.	4-6/e.f.	1-3/s.f.
11	1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
12	1-3/s.f.		1-3/e.f.	1-3/e.f.
13	1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
14	1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.

E: red blood cell

Pre	0 h	1.5 h	3 h
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.		1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/e.f.
1-3/s.f.		1-3/e.f.	1-3/e.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.
1-3/s.f.	1-3/s.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.		1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/e.f.	1-3/s.f.
1-3/s.f.	1-3/e.f.	1-3/s.f.	1-3/s.f.

A-renal tubular epithelial cell
B-renal tubular epithelial cast
C-granular cast: Data are 1-3 counts by several field (/s. f.), 1-3 counts by every field (/e. f.)
D-white blood cell: Data are 1-3 counts by several field (/s. f.), 1-3, 4-6, 7-10, 11-15 and 16-20 counts by every field (/e. f.)
E: red blood cell: Data are 1-3 counts by several field (/s. f.), 1-3 counts by every field (/e. f.)
Urinary sediments were analyzed by microscope (x400 high powerd field)

after exercise in the damaged group. Thus, the degree of renal disorder of Ccr, urinary excretion rates of ALB and urinary excretion rate of NAG activity in the damaged group were higher than the minor-damaged group.

Plasma and urine cytokines in relation with renal damage

In both groups, plasma concentrations of IL-1ra, IL-6, IL-8 and MCP-1 increased significantly after the race compared with the pre-exercise values. Plasma IL-10 concentration increased significantly at 0 h after exercise compared with the pre-exercise value in the damaged group only. However, urinary excretion rates of IL-8, IL-10 and IFN-γ increased significantly, and urinary excretion rates of IL-2

Table 3. Criteria of AKI

RIFLE

	Cr/GFR Criteria	Urine Output (UO) Criteria
Risk	Increased Cr ×1.5 or GFR decreases ＞25%	UO ＜0.5ml/kg/hr ×6hr
Injury	Increased Cr ×2 or GFR decreases ＞50%	UO ＜0.5ml/kg/hr ×12hr
Failure	Increased Cr ×3 or GFR decreases ＞75% or Cr ≧4mg/dl (with acute rise of ≧0.5mg/dl)	UO ＜0.3ml/kg/hr ×24hr or anuria ×12hr
Loss	Persistent ARF = Complete loss of renal function for ＞4weeks	
ESRD	End Stage Renal Disease	

AKIN

	Cr Criteria	Urine Output (UO) Criteria
Stage1	Increased Cr ×1.5 or ≧0.3mg/dl	UO ＜0.5ml/kg/hr ×6hr
Stage2	Increased Cr ×2	UO ＜0.5ml/kg/hr ×12hr
Stage3	Increased Cr ×3 or Cr ≧4mg/dl (with acute rise of ≧0.5mg/dl)	UO ＜0.3ml/kg/hr ×24hr or anuria ×12hr

Modified by Cruz et al. Critical care, 13:211.2009 (6)

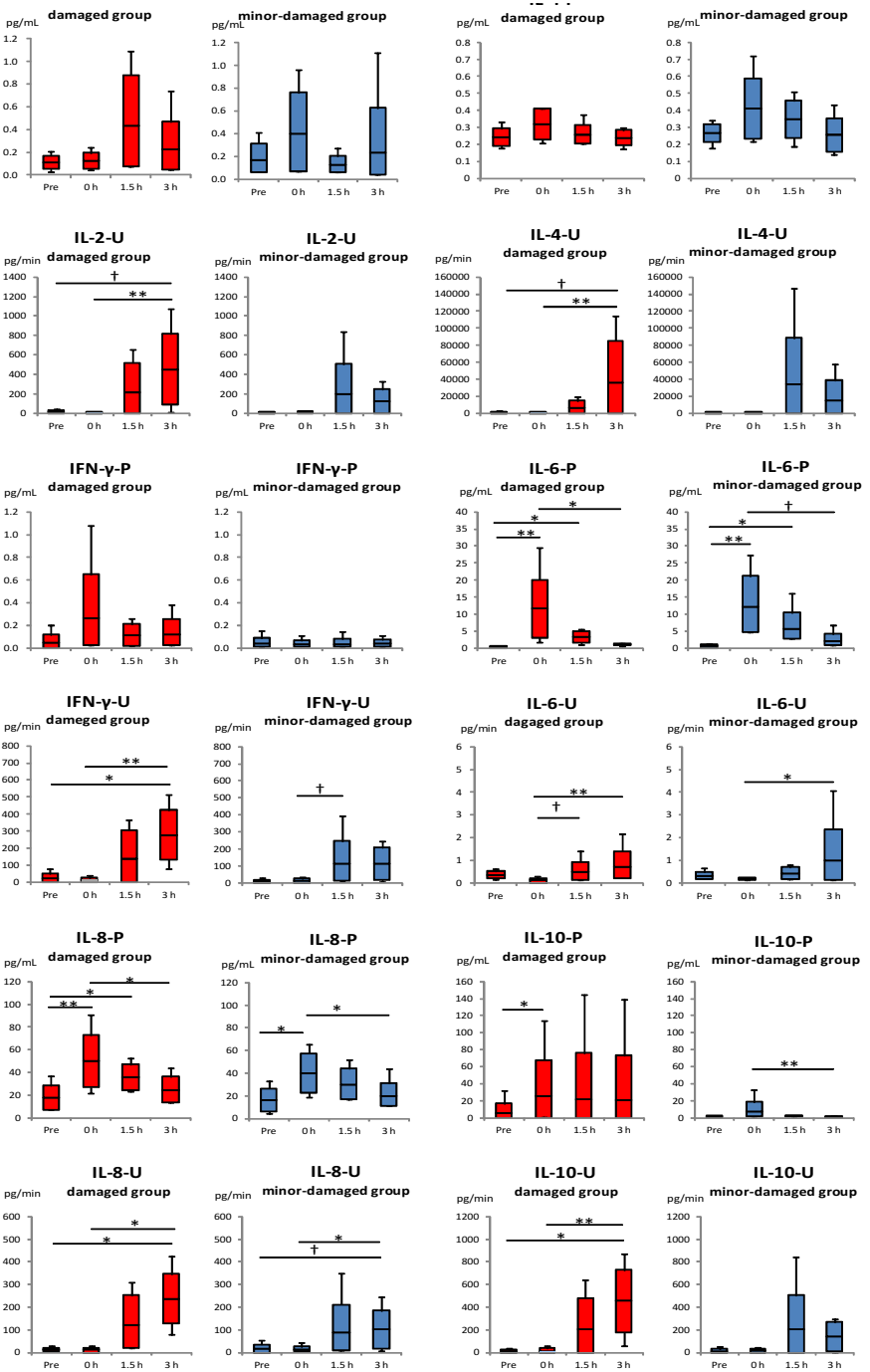
Abbreviations: Risk, Injury, Failure, Loss, End Stage Kidney Disease (RIFLE), acute kidney injury network (AKIN), creatinine (Cr), glomerular filtration rate (GFR), acute renal failure (ARF).

and IL-4 tended to increase after the race compared with the pre-exercise values in the damaged group. There was a trend for urinary excretion rate of IL-8 to increase following exercise in the minor-damaged group. In both groups, urinary excretion rates of IL-6 were significantly elevated at 3 h after the race as compared to 0 h-post exercise values. In the damaged group, urinary excretion rates of IL-2, IL-4 and MCP-1 increased significantly at 3 h after the race compared with 0 h-post exercise. On the other hand, urinary excretion rates of TNF-α, IL-1β, IL-1ra and IL-12 did not change significantly after the race in both groups (Figure 1, Table 4).

Table 4. Changes of urine volume and cytokines following the duathlon race.

	Renal tubular epithelial cell	Unit	Pre	0 h	1.5 h	3 h	Fried-man test	Scheffe test
Urine volume	(+) n=7	mL/min	1.8 ± 0.84	0.31 ± 0.22	0.79 ± 0.36	1.5 ± 0.95	**	Pre-0 h** 0 h-3 h†
	(-) n=7		1.9 ± 1.0	0.62 ± 0.41	1.1 ± 0.59	2.9 ± 3.0	†	Pre-0 h†
TNF-α-P	(+)	pg/mL	0.40 ± 0.61	0.11 ± 0.03	0.22 ± 0.22	1.6 ± 2.3	-	-
	(-)		0.23 ± 0.25	0.10 ± 0.05	0.30 ± 0.34	0.28 ± 0.29	-	-
TNF-α-U	(+)	pg/min	3.7 ± 4.4	0.61 ± 0.53	2.7 ± 2.3	5.6 ± 8.4	-	-
	(-)		3.7 ± 3.3	2.0 ± 1.4	2.9 ± 4.4	3.4 ± 4.0	-	-
IL-1ß-P	(+)	pg/mL	0.09 ± 0.15	0.70 ± 1.7	1.4 ± 2.8	0.23 ± 0.24	-	-
	(-)		0.03 ± 0.08	0.16 ± 0.38	0.49 ± 0.83	0.35 ± 0.50	**	Pre-0 h† Pre-1.5 h† Pre -3 h*
IL-1ß-U	(+)	pg/min	2.1 ± 2.0	1.2 ± 1.3	8.6 ± 11.2	4.8 ± 7.0	-	-
	(-)		1.2 ± 0.74	3.2 ± 3.0	1.8 ± 2.4	1.2 ± 1.2	*	-
IL-1ra-P	(+)	pg/mL	54.7 ± 52.1	3545 ± 7386	9391 ± 8791	3846 ± 3996	**	Pre-1.5 h** Pre -3 h†
	(-)		46.3 ± 45.1	6572 ± 9398	7057 ± 9124	2822 ± 5300	**	Pre-0 h** Pre -1.5 h**
IL-1ra-U	(+)	pg/min	186.8 ± 163.5	175.4 ± 360.4	8328 ± 17263	2500 ± 4362	†	-
	(-)		293.7 ± 244.8	1556 ± 2712	2251 ± 2663	3781 ± 8695	-	-
L-12-P	(+)	pg/mL	0.21 ± 0.07	0.30 ± 0.12	0.24 ± 0.09	0.22 ± 0.07	-	-
	(-)		0.20 ± 0.09	0.29 ± 0.21	0.27 ± 0.17	0.20 ± 0.09	-	-
IL-12-U	(+)	pg/min	2.6 ± 2.9	1.0 ± 1.2	2.6 ± 1.8	3.2 ± 4.3	-	-
	(-)		2.4 ± 1.3	1.9 ± 0.67	1.8 ± 2.5	3.5 ± 5.6	-	-

Values: means ± SD (n=7). Statistics: **p < 0.01, *p < 0.05, †p < 0.1.
-P: Data are plasma concentration. -S: Data are serum concentration.
-U: Data are the gross amount in the volume of urinary excretion per one minute.
pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points.
(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.
(-): minor-damaged group; renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: tumour necrosis factor (TNF), interleukin (IL), IL-1 receptor antagonist (IL-1ra).


damaged group
minor-damaged group
damaged group
minor-damaged group
IL-2-U
damaged group
IL-2-U
minor-damaged group
IL-4-U
damaged group
IL-4-U
minor-damaged group
IFN-γ-P
damaged group
IFN-γ-P
minor-damaged group
IL-6-P
damaged group
IL-6-P
minor-damaged group
IFN-γ-U
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IL-6-U
minor-damaged group
IL-8-P
damaged group
IL-8-P
minor-damaged group
IL-10-P
damaged group
IL-10-P
minor-damaged group
IL-8-U
damaged group
IL-8-U
minor-damaged group
IL-10-U
damaged group
IL-10-U
minor-damaged group
pg/mL
pg/min
Pre
0 h
1.5 h
3 h

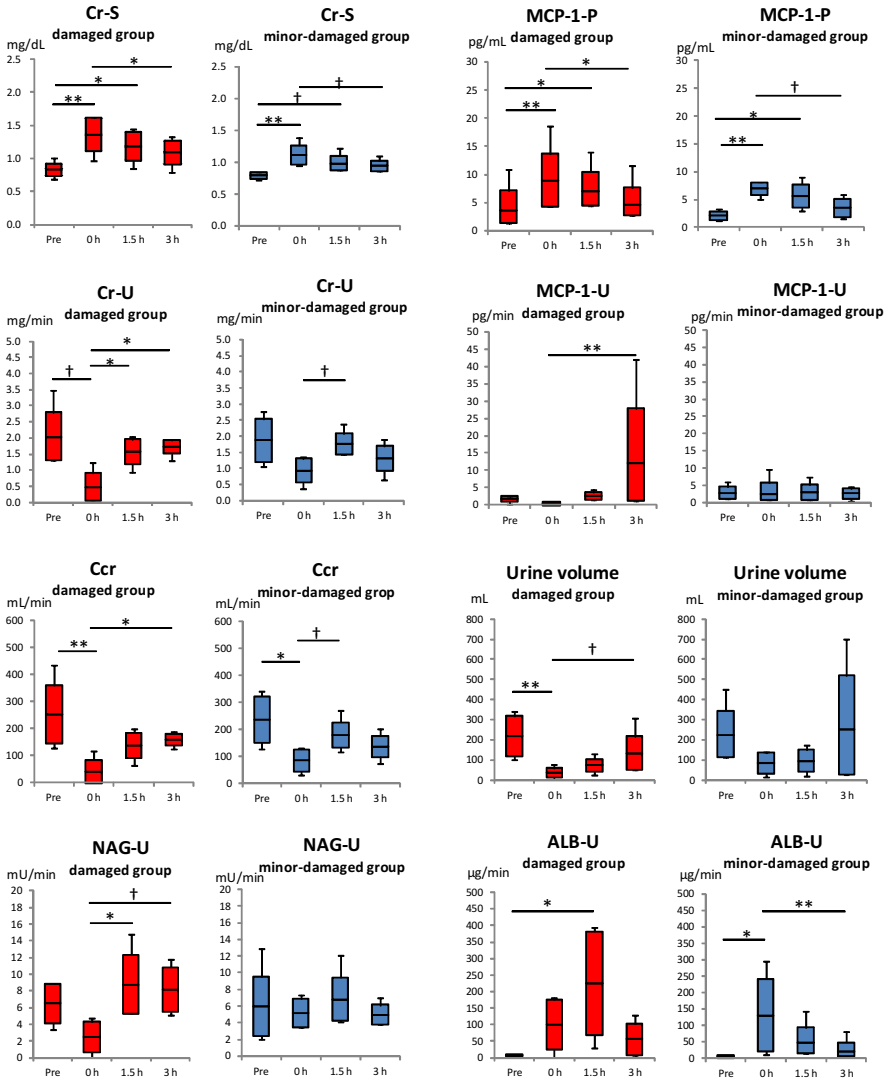

Figure 1. Changes of cytokines and parameters of renal damage following the duathlon race. pre-exercise (Pre), immediately post-exercise (0 h), 1.5 hour post-exercise (1.5 h) and 3 hour post-exercise (3 h) are sampling points.
damaged group: renal tubular epithelial cells existed in the urinary sediments.
minor-damaged group: renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: creatinine (Cr), creatinine clearance (Ccr), albumin (ALB), *β-N*-acetyl-D-glucosaminidase (NAG) activity, interleukin (IL), interferon (IFN), monocyte chemotactic protein (MCP)-1.
Values: means ± SD (n=7). Statistics: *$p < 0.05$, **$p < 0.01$, †$p < 0.1$.
Box plot: minimum values means SD means means SD maximum values.
-P: Data are plasma concentration.
-S: Data are serum concentration.
-U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).

DISCUSSION

Studies investigating changes in the urinary levels of cytokines several hours after exhaustive endurance exercise have not yet been reported. In the present study, we present new evidence that changes in the urinary excretion of some cytokines were much greater than plasma cytokine changes, and might provide sensitive biomarkers of responses to exercise. IL-8 and IL-10 increased in both plasma and urine after exercise. In contrast, IL-1β, IL-1ra, IL-6 and MCP-1 mainly increased in plasma, whereas IL-2, IL-4 and IFN-γ only increased in urine after exercise. These results suggest that following exercise IL-1β, IL-1ra and MCP-1 were cleared slowly from the circulation with the plasma levels reflecting the systemic release, whereas IL-2, IL-4 and IFN-γ were rapidly excreted from the circulation into urine, without any increase in the systemic circulation, or the kidney might be the source of these cytokines as discussed later.

TNF-α has been considered as a primary mediator of the SIRS response, but the plasma and urine concentrations of TNF-α did not change significantly in the present study. On the other hand, the plasma concentration of IL-6 increased significantly by more than 30-fold immediately after the race, which is consistent with previous findings (4, 9, 14, 28-30, 32-34, 37, 46). However, the urine concentration of IL-6 did not change markedly—especially when corrected for urinary creatinine clearance. Although the plasma concentration of IL-1β increased significantly 1.5 h after the race, this response occurred later than the IL-6 response, and the concentration of IL-1β was relatively lower than IL-1ra, indicating that the cascade of cytokine production during exercise differs from the classical pro-inflammatory cytokine cascade that occurs during acute inflammation such as sepsis (1, 10, 19, 33, 37, 46). Chemokines are also considered important in the pro-inflammatory process. IL-8 is a potent neutrophil chemotactic and activation protein, whereas MCP-1 promotes monocyte activation and extravasation into inflammatory tissues (22). The plasma concentrations of IL-8 and MCP-1 increased significantly immediately after the race in the present study, which is also consistent with previous studies (24-27, 32-35, 44-49). Although exhaustive exercise enhances the capacity of neutrophils to produce reactive oxygen species (27, 43, 44), which is also one of the main pathogenic mechanisms of multiple organ failure in SIRS (1), these chemokines may, at least in part, mediate such exercise-induced pathogenesis as muscle inflammatory damage, exertional rhabdomyolysis and heat-related multiple organ failure (27, 44-46).

In contrast to these pro-inflammatory cytokines, we demonstrated that plasma IL-1ra concentration increased by more than 170 times in the present study. We have previously shown an increase of IL-1ra by 214-fold after a marathon race (45). IL-1ra is a natural antagonistic cytokine that competes with IL-1 for receptor binding without inducing signal transduction. Regardless of whether IL-1β is released in a small quantity after exercise in a delayed-onset manner, the higher concentration of IL-1ra appears to block the IL-1 bioactivity in advance—at least in the circulation. In the present study, IL-10 increased not only in plasma (5-fold) but also in urine by more than 50-fold. IL-10 causes immunosuppression associated with various forms of trauma by attenuating IL-1β, TNF-α, IFN-γ, IL-6 and IL-8 and enhancing IL-1ra production (16, 33, 52). There was no increase in plasma IL-4, but we observed a significant increase in urinary concentration of

IL-4 after exercise in the present study. In support of the present findings, we have previously shown no changes in plasma IL-4 concentration following a marathon (45). However, we have reported an increase in urinary IL-4 concentrations 2 h after short-duration, maximal exercise (46). The 350-fold increase in urinary IL-4 concentration in the present study is, to our knowledge, the most dramatic change reported among existing studies on exercise and cytokines. IL-4 has been shown to down-regulate pro-inflammatory cytokines and up-regulate IL-1ra production (52). In view of the large changes and biological characteristics of these anti-inflammatory cytokines, it is possible that they play a role in immunosuppression and increased susceptibility to infection after endurance exercise (3, 23, 39). IL-4 is also a strong inducer of type-2 cytokines such as IL-6 and IL-10, and its involvement in patients with atopic disposition and past history of exercise-induced allergy and anaphylaxis seems worthy of further investigation (13, 40).

We did not collect blood samples during exercise, so it is uncertain whether IL-2 was elevated and active within the bloodstream during exercise. IL-2 may bind to soluble receptors, and/or act locally within lymphoid tissues (36), which could explain why it did not appear in plasma following exercise (11, 46). We observed that plasma concentration of IFN-γ was low, whereas urinary excretion of both IL-2 and IFN-γ was large following exhaustive exercise in this study. Although one study reported the urinary excretion of IFN-γ after 20-km running (38), other groups have not reported any change (11, 29, 53). Several factors might explain these inconsistencies. Firstly, we used a different manufacturer's ELISA kit from the previous studies that may capture a different epitope or have enhanced sensitivity. Secondly, the exercise conditions and time course of sampling points especially for the recovery period in the present study were different from previous studies. Lastly, other investigators have not standardized fluid intake or corrected for glomerular filtration by expressing cytokine concentrations relative to urinary creatinine content. Although we observed, somewhat unexpectedly, that the post-exercise urinary concentrations of IL-2, IL-4, IL-8, IL-10 and IFN-γ were several orders of magnitude higher compared with plasma, this might be because we provided fluid replacement during exercise to prevent dehydration and augment urine output with enhanced renal clearance and reduced re-absorption, or the kidney in itself might be the main source of the cytokine production. In any case, the present data demonstrate that IL-2, IL-4 and IFN-γ were produced in the body following exhaustive endurance exercise.

In this study, according to the values of serum Cr in the AKI diagnosis criteria such as “Risk, Injury, Failure, Loss, End Stage Kidney Disease (ESKD): RIFLE criteria” (2) and “acute kidney injury network: AKIN” (18, 20) (Table 3), AKI following endurance exercise showed “Risk” or “Stage1”at 0 h and 1.5 h after the race in the damaged group and at 0 h after exercise in the minor-damaged group. Moreover, the degree of renal disorder of Ccr, urinary excretion rate of ALB and urinary excretion rate of NAG activity in the damaged group were higher than the minor-damaged group. In the present study, the relations among the changes of plasma and urine levels of various cytokines and AKI were investigated. In both groups, plasma concentrations of IL-2, IL-4 and IFN-γ did not change significantly after the race as compared with the pre-exercise values whereas urinary excretion of IFN-γ increased significantly and urinary excretion

of IL-2 and IL-4 tended to increase after the race compared with the pre-exercise values in the damaged group only. Therefore, it might be possible that IL-2, IL-4 and IFN-γ were produced in the kidney that caused AKI following endurance exercise. Moreover, urinary excretion of IL-8 and IL-10 also increased significantly after the race as compared with the pre-exercise values in the damaged group only, but plasma concentrations of IL-8 and IL-10 also increased significantly post-exercise when compared with the pre-exercise in both groups of IL-8 and in the damaged group of IL-10. In the damaged group only, urinary excretion rates of MCP-1 increased significantly at 3 h after the race as compared with 0 h-post exercise values. However, urinary excretion of MCP-1 was increased until 3 h-post exercise. Furthermore, urinary excretion of IL-8, IL-10 and MCP-1 in the damaged group was elevated markedly more than the ones in the minor-damaged group. Accordingly, it might be possible that IL-8, IL-10 and MCP-1 were produced in the circulation and/or kidney, which caused AKI following endurance exercise.

The present data suggest that although some cytokines may not appear in the circulation during exercise, they do appear in urine after several hours following endurance exercise, which indicates their production and/or clearance from the bloodstream in the kidney. These findings might help to develop reliable biomarkers of immunity and inflammation for the assessment of endurance exercise workload as well as the pathological mechanisms of strenuous exercise. Further research is needed to determine the mechanisms of the production and clearance of the cytokines in the kidney from the circulation following prolonged endurance exercise. Although the wider physiological and pathological implications are still not clearly understood, such cytokine kinetics may partly explain the exercise-induced suppression of cell-mediated immunity (3, 23, 34, 36, 39), organ damage and inflammation (4, 14, 29, 32-34, 37, 43-49), and increased allergic reactions (13, 40, 46). The relationships among these changes, their clinical significance and the possibility of intervention must be clarified in future research.

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