

Effects of strong physical exercise on blood glutamate and its metabolite 2-ketoglutarate levels in healthy volunteers

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Excessive concentrations of L-glutamate (glutamate) have been found to possess neurotoxic properties. This study investigates how stress induced by strong physical exercise affects blood glutamate, 2-ketoglutarate, Alanine aminotransferase (ALT) and Aspartate Aminotransferase (AST) levels. The relationship between muscle damage caused by strong physical exercise and blood glutamate levels was also examined. Twenty-two healthy volunteers engaged in intense veloergometry (“spinning”) for a duration of 60 minutes. Two 10 minute peaks of extremely intense exercise were performed at 10 minutes and 50 minutes after the start of exercise. After 60 minutes of exercise, volunteers were monitored for an additional 180 minutes in resting conditions. Blood samples for determination of glutamate and 2-ketoglutarate levels were collected prior to exercise and then every 30 min for entire experiment. Blood samples were also taken at those time points to measure glutamate, 2-ketoglutarate, AST, ALT, creatine phosphokinase (CPK), myoglobin, lactate and venous blood gas levels. Blood glutamate levels were significantly elevated throughout the exercise session ($P < 0.001$) and then returned to baseline levels at the cessation of exercise. 2-ketoglutarate, a product of glutamate metabolism, reached significantly elevated levels at 30 minutes ($P < 0.01$) from the start of exercise and remained elevated up to 240 minutes post exercise initiation ($P < 0.001$). AST and ALT levels were elevated at 60 minutes when compared to baseline. AST levels remained elevated at 240 minutes, unlike ALT levels which returned to baseline values at 240 minutes. Strong physical exercise leads to a significant elevation in blood glutamate, most likely as a result of skeletal muscle damage. 2-ketoglutarate was also found to be elevated for long periods of time, reflecting an ongoing process of glutamate breakdown. Elevated concentrations of AST and ALT in plasma reflect the importance of these enzymes in the maintenance of stable blood glutamate concentrations.

Key words: exercise, glutamate, hyperthermia, muscle damage, neurotoxicity, stress

INTRODUCTION

Several acute and chronic brain insults have been associated with high L-glutamate (glutamate) concentrations in brain extracellular fluid (ECF) and cerebrospinal fluid (CSF) such as stroke, traumatic brain injury (TBI), subarachnoid hemorrhage (SAH) and meningitis (Castillo et al. 1996, 1997, Davalos et al. 1997, Johnston et al. 2001, Serena et al. 2001, Castillo

et al. 2002, Vesce et al. 2007). The presence of glutamate in brain ECF following ischemic insult has been shown to correlate with poor neurological outcomes, in both animal models (Palmer et al. 1993, Camacho and Massieu 2006) and in human clinical studies (Baker et al. 1993, Zauner et al. 1996, Bullock et al. 1998, Koura et al. 1998, Yamamoto et al. 1999, Zhang et al. 2001, Rose et al. 2002, Richards et al. 2003).

Two major pathways for the removal of excess glutamate from brain interstitial fluids have been described. The more commonly described mechanism involves the uptake of glutamate by excitatory amino acid transporters found on neuronal and glial cells

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(Danbolt 2001, Cohen-Kashi-Malina et al. 2012). However, excess glutamate is also removed by the diffusion of glutamate from brain ECF to blood, following a brain to blood glutamate gradient (Berl et al. 1961). Transporters for glutamate have been identified on the antiluminal side of endothelial cells in brain capillaries. These transporters are thought to assist in the uptake of glutamate into the capillary cells, hence building a favorable gradient for the diffusion of glutamate from capillaries into the blood (O'Kane et al. 1999).

Several studies have reinforced the notion that blood glutamate levels have an important impact on brain glutamate levels and influence clinical outcomes. Scavenging blood glutamate by converting glutamate into its inactive metabolite, 2-ketoglutarate, has been shown to increase the flow of glutamate from CSF and ECF into blood and improve neurological outcomes after TBI in rats (Zlotnik et al. 2007, 2008, 2009, 2012a,b). Pyruvate and oxaloacetate are co-substrates for the blood resident enzymes Alanine aminotransferase (ALT) and Aspartate transaminase (AST), respectively, which convert glutamate into 2-ketoglutarate (Gottlieb et al. 2003). Moreover, intravenous injection of glutamate into blood has been shown to decrease the driving force of glutamate out of the brain, and correlates with a poor neurological outcome following TBI in rats (Zlotnik et al. 2007, 2008, 2009, Teichberg et al. 2009).

Previous studies have shown that there is a rapid decline in blood glutamate levels following closed head injury (CHI) in rats (Zlotnik et al. 2007, 2010, Helms et al. 2012). This phenomenon has been shown to be partially attributed to a stress response, *via* a glutamate-reducing effect of the activation of β 2 adrenergic receptors (Zlotnik et al. 2010). In this way the body attempts to facilitate the clearance of excess glutamate from brain ECF in order to promote a better neurological outcome. Animal stress models have consistently demonstrated a rapid elevation of cellular enzymes AST, ALT, creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) following adrenergic stress, correlating with the rise in plasma catecholamine levels (Mukherjee and Ghosh 1987, Sen et al. 1992, Arakawa et al. 1997). When pharmacologic sympathectomy is preformed, the enzyme surge is depressed, while selectively blocking alpha receptors shows a near normal response. These findings suggest that the elevation in enzymes seen in stress may be

attributable to beta-adrenergic activation. In accord with findings in lab animals, human studies of stress reveal an elevation of AST and ALT following stress and anaerobic metabolism, such as in adolescent athletes and soldiers under physical stress (Kayashima et al. 1995, Fu et al. 2002). While the rise in transaminases after marathon stress may be attributable to adrenergic stimulation, other processes, such as muscle breakdown may contribute as well. In a study performed in marathon runners, AST, CPK and myoglobin rise significantly at 4 hours post marathon. At 24 hours post marathon, AST remains elevated compared to baseline, but is significantly lower compared to 4 hours. This phenomena may be attributable both to muscle breakdown or to adrenergic stimulation due to the intense exercise (Kratz et al. 2002).

Several studies have implicated that athletic activity, particularly soccer and US football may be associated with development of sporadic amyotrophic lateral sclerosis (ALS) (Chio et al. 2009, Sutedja et al. 2009, Stern et al. 2011). While the precise mechanism leading to ALS is not completely clear, elevated glutamate concentrations may be involved by elevation of D-serine and directly activating NMDA receptors (Paul and de Bellerroche 2012).

Another possible mechanism involved in blood glutamate homeostasis includes shifting of glutamate in and out of peripheral tissues serving as glutamate depots. Recently, using a radiolabeling technique we have found that glutamate redistributes rapidly in an exponential fashion, accumulating most significantly in skeletal muscle which contributes 59% of the total body capacity (Klin et al. 2010). Conversely, different types of stress in humans, including physical stress and elevation of stress hormones, have been shown to deplete muscle glutamate content, causing a transient modest rise in plasma glutamate and a subsequent rapid decrease (Hammarqvist et al. 2001, Blomstrand and Essen-Gustavsson 2009).

The purpose of this study was to investigate the effects of stress in healthy human volunteers on blood glutamate homeostasis and to elucidate the physiologic mechanisms involved in the elimination of excessive glutamate from plasma. Taking into account two counteracting mechanisms involved: (1) release of glutamate from depots into blood; (2) glutamate reducing effects of stress, we hypothesized that along the course of exercise these processes will act to either increase or reduce blood glutamate concentrations. Specifically,

we examined how stress induced by strong physical exercise affects blood glutamate, 2-ketoglutarate, AST and ALT levels. The relationship between muscle damage caused by strong physical exercise and blood glutamate levels was also examined.

METHODS

This experiment has been conducted according to recommendations by the Helsinki committee and was approved by the Ethics Committee of Ben Gurion University of the Negev, Beer Sheva, Israel. Written informed consent was obtained from each participant before beginning the experiment.

Exercise protocol

Twenty-two healthy male volunteers engaged in intense veloergometry ("spinning") for the duration of 60 minutes. The exercise session was led and supervised by a certified spinning instructor. Two 10 minute peaks of extremely intense exercise were performed at 10 minutes and 50 minutes after the start of exercise. After 60 minutes of exercise, volunteers were monitored for an additional 180 min in resting conditions. Subjects were allowed to drink water throughout the protocol.

Physiological monitoring

Volunteers were intermittently monitored for heart rate and noninvasive blood pressure every 30 min throughout the entire experiment. These values were used as markers of intensity and stress. Temperature was measured before exercise and at 30, 60, 120 and 240 minutes after the start of exercise.

Blood analysis

All volunteers had an 18G IV catheter inserted to either their right or left antecubital vein. Blood was drawn prior to exercise initiation (designated as baseline values) as well as every 30 minutes throughout the experiment for determination of glutamate, 2-ketoglutarate and glucose. Blood for arterial blood gas analysis was collected into heparinized 2 cc syringes at 0, 60 and 240 minutes and was immediately analyzed in the biochemical laboratory of Soroka Medical Center. Blood samples for the determination of

Myoglobin and CPK were collected at 0, 60 and 240 minutes and measured as a surrogate indicator of muscle damage in the biochemical laboratory of Soroka Medical Center (Olympus AU 2700). Lactate levels served as a marker of efficiency of physical exercise and anaerobic metabolism. Blood glucose level was determined immediately after collecting each blood sample with a "glucocheck" blood glucose monitoring device (Accutrend Sensor Roche, USA). AST and ALT levels were determined in the biochemical laboratory of Soroka Medical Center via a fluorescent method, based on conversion of glutamate into alanine and aspartate in the presence of AST and ALT respectively (Olympus AU 2700) at 0, 60 and 240 minutes. Glutamate and 2-ketoglutarate were analyzed according to the following protocol. Whole blood (200 μ l aliquot) was deproteinized by adding an equal volume of ice-cold 1M perchloric acid (PCA) and then centrifuging at $10\,000\times g$ for 10 minutes at 4°C . The pellet was discarded and supernatant collected, adjusted to pH 7.2 with 2M K_2CO_3 and, if needed, stored at -80°C for later analysis. Glutamate concentration was measured using the fluorometric method of Graham and Aprison (Graham and Aprison 1966). A 20 μ l aliquot from the PCA supernatant was added to 480 μ l of a 0.3 M glycine, 0.25 M hydrazine hydrate buffer adjusted to pH 8.6 with 1M H_2SO_4 and containing 15 U of glutamate dehydrogenase in 0.2 mM NAD. After incubation for 30–45 minutes at room temperature, the fluorescence was measured at 460 nm with excitation at 350 nm. A glutamate standard curve was established with concentrations ranging from 0–400 μM at 6 different points. All determinations were done at least in duplicates.

Statistical analysis

Statistical analysis was performed using SPSS 17 software. Data are presented as mean \pm SEM. It was hypothesized that the glutamate, AST, ALT, concentrations in blood would initially rise, and at some point decline back toward baseline. AST and ALT were expected to correlate with the concentrations of blood glutamate. Blood pressure, pulse and temperature were assumed to change over the course of exercise as well. Parametric data were assessed using a repeated measures analysis of variance with Bonferroni *post hoc* testing and a paired *t*-test was used when applicable.

RESULTS

We found glutamate 2-ketoglutarate, AST and ALT to be normally distributed in our study group.

Participants

22 male volunteers participated in the study. The average age was 31.5 ± 6 years. BMI was 23.2 ± 3.4 . None of the volunteers had any significant chronic disease, and none adhered to a strict or extreme diet.

Blood glutamate concentration

Blood glutamate levels were significantly elevated 30 minutes after the initiation of exercise (193 ± 21 $\mu\text{M/L}$ vs. 232 ± 25 $\mu\text{M/L}$, $P < 0.001$), remained elevated throughout the exercise session and returned to baseline in the time interval between 90 and 120 minutes after the start of exercise (corresponding to 30–60 minutes after exercise cessation). Blood glutamate levels are presented in Figure 1. Elevation of blood glutamate levels correlated with the timing of intense exercise and diminished rapidly upon cessation of exercise.

Blood 2-ketoglutarate levels

2-ketoglutarate, an end product of glutamate metabolism, reached significantly elevated levels at 30 min-

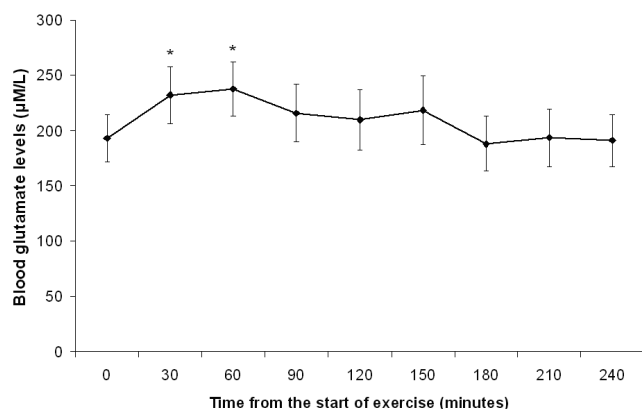


Fig. 1. Blood glutamate levels at different time points throughout the exercise session. At 30 and 60 minutes after the start of exercise, glutamate levels were significantly elevated ($*P < 0.001$). Blood glutamate returned to baseline levels by 90 minutes after exercise initiation. Data is presented as mean \pm SEM.

utes ($P < 0.01$) from the start of exercise. Levels gradually increased and remained elevated up to 240 minutes after exercise initiation (corresponding to 180 minutes post-exercise cessation) ($P < 0.001$). 2-ketoglutarate levels are presented in Figure 2. In comparison to the elevation of glutamate, the elevation in 2-ketoglutarate was longer-lasting, reflecting the continuous breakdown of glutamate and the conversion into its inactive metabolite 2-ketoglutarate throughout the duration of glutamate leak from muscle tissue.

Blood AST and ALT

Blood resident enzymes AST and ALT, in conjunction with their co-substrates oxaloacetate and pyruvate, are involved in the metabolism of glutamate into 2-ketoglutarate. Therefore, an increase in blood glutamate concentration followed by a subsequent decline in blood glutamate concentration is expected to correlate with high levels of AST and ALT, with the co-existing formation of 2-ketoglutarate as a result of glutamate breakdown.

AST and ALT concentrations were assessed at 0, 60, 240 minutes from the start of exercise and are presented in Table I. AST and ALT levels were elevated at 60 minutes when compared to baseline. AST levels remained elevated at 240 minutes, unlike ALT levels which returned to baseline values at 240 minutes. The

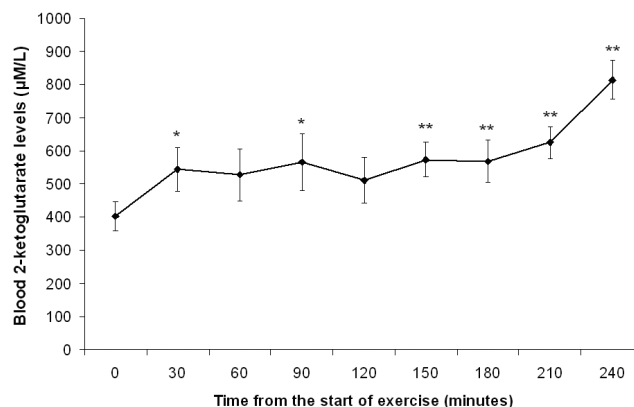


Fig. 2. Blood 2-ketoglutarate levels at different time points throughout the exercise session. 2-ketoglutarate levels reached significantly elevated levels at 30 min and 90 min ($*P < 0.01$) from the start of exercise. Levels gradually increased and reached significantly elevated levels at 150 min from the start of exercise and remained elevated up to 240 min post exercise initiation ($**P < 0.001$). Data is presented as mean \pm SEM.

elevation in ALT and AST coincides with the peak in blood glutamate levels, establishing their role in glutamate metabolism.

Blood glucose

Blood glucose levels remained constant and no significant changes were noted throughout the exercise. Data not shown.

Lactate

Elevated lactate levels reflect anaerobic metabolism in muscle tissue, which in turn represents muscle stress and damage. Lactate increased significantly by 60 minutes, returning to baseline by 240 minutes (Table II).

Venous blood gasses

PvCO₂, pH, PvO₂, and HCO₃ were analyzed at baseline, as well as 60 and 240 minutes after the start of exercise (Table II). As expected, the intense physical activity caused subjects to hyperventilate, demonstrated by an increase in PvO₂ compared to baseline (45 ± 15.3 mmHg vs. 72 ± 15.2 mmHg, $P < 0.001$), and a decrease in PvCO₂ (46 ± 7 mmHg vs. 32 ± 5 mmHg, $P < 0.001$). Compared to baseline, pH at 60 minutes decreased (7.36 ± 0.037 vs. 7.33 ± 0.041, $P < 0.001$) despite hyperventilation, as a result of increased anaerobic metabolism and lactate production.

Table I

Blood concentrations of enzymes AST and ALT over the course of the exercise session

	<i>t</i> =0 min	<i>t</i> =60 min	<i>t</i> =240 min
AST (IU/L)	22 ± 2	25 ± 2*	24 ± 2*
ALT (IU/L)	16 ± 2	19 ± 2*	17 ± 2

Both AST and ALT are significantly elevated at 60 minutes compared to baseline (* $P < 0.05$). AST remains elevated at 240 minutes (* $P < 0.05$) while ALT returned to baseline levels at 240 minutes. Data is presented as mean ± SEM.

Myoglobin and CPK

Myoglobin and CPK were used as markers of muscle damage, which may explain glutamate leakage from muscle tissue. CPK and Myoglobin values both increased by 90 minutes and remained elevated by 240 minutes (Table III).

Arterial blood pressure and heart rate

Mean arterial blood pressure increased transiently, peaking at 30 minutes (baseline vs. 30 minutes 96 ± 3 vs. 106 ± 5 mmHg, $P < 0.01$), and started to consistently decline at 90 minutes after initiation of exercise ($P < 0.001$), reaching lower than baseline levels. Heart rate was increased at 30, 90, 120

Table II

Venous blood gasses at baseline, 60 and 240 minutes after the start of exercise

	<i>t</i> =0 min	<i>t</i> =60 min	<i>t</i> =240 min
pH	7.36 ± 0.01	7.33 ± 0.01*	7.35 ± 0.01
PvCO ₂ (mmHg)	46 ± 1.58	32 ± 1.2**	45 ± 1.42
PvO ₂ (mmHg)	45 ± 3.36	72 ± 3.34**	38 ± 3.54
HCO ₃ (meq/L)	24.3 ± 0.25	19.1 ± 0.69**	23.1 ± 0.32*
Lactate (mg/dl)	1.4 ± 0.09	7.9 ± 0.82**	1.78 ± 0.3

All values show significant changes between baseline resting values and those obtained immediately at termination of exercise. Aside from HCO₃, all measured parameters return to their resting values at 240 minutes. Data is presented as mean ± SEM. * $P < 0.05$; ** $P < 0.001$.

Table III

Myoglobin and CPK values throughout the exercise session			
	<i>t</i> =0 min	<i>t</i> =60 min	<i>t</i> =240 min
CPK (U/L)	98 ± 8	130 ± 15*	185 ± 30**
Myoglobin (µg/L)	37 ± 2	49 ± 4*	86 ± 22*

These markers of muscle damage rise significantly by 90 minutes and remain elevated by 240 minutes. Data is presented as mean ± SEM. **P*<0.05; ***P*<0.01.

(*P*<0.001) and 150 (*P*<0.01) and returned to baseline levels at 180 minutes after the start of exercise. Heart rate was measured to be elevated again at 240 minutes (*P*<0.01). Values for the mean arterial blood pressure and heart rate during the exercise session are seen in Table IV.

Body temperature

Core body temperature remained unchanged throughout the length of the experiment. Data not shown.

DISCUSSION

The principal finding in this study was that strong physical exercise led to a significant elevation in blood glutamate, most likely as a result of skeletal muscle damage. However, participants were capable of maintaining a stable blood glutamate concentration despite a continuous leak of fresh glutamate from the injured

muscle, as levels returned to baseline values soon after the cessation of physical exercise. This finding reflects the importance of the body to maintain blood glutamate concentrations within normal physiological ranges. 2-ketoglutarate was also found to be elevated for long periods of time, reflecting an ongoing process of glutamate breakdown. Lastly, elevated concentrations of AST and ALT in plasma reflect the importance of these enzymes in the maintenance of stable blood glutamate concentrations.

Elevated glutamate levels in brain ECF and CSF has been associated with a wide variety acute and chronic brain disease states, including stroke and TBI (Castillo et al. 1996, 1997, 2002, Davalos et al. 1997, Johnston et al. 2001, Serena et al. 2001, Vesce et al. 2007). Moreover, there is a tight correlation between the elevated levels of glutamate and a poor neurological outcome (Zauner et al. 1996, Koura et al. 1998, Zhang et al. 2001, Zlotnik et al. 2012b), suggesting that the removal of excess glutamate may possibly improve the neurological outcome after CHI. According to findings of this study, one may speculate that repeated athletic activity, leading to frequent rises in blood glutamate, may have a role in the poorer neurological outcomes found in professional athletes found in some studies. Athletic activity, particularly soccer and US football may be associated with development of sporadic amyotrophic lateral sclerosis (ALS) a condition associated with deterioration of upper and lower motor neurons (Chio et al. 2009, Sutedja et al. 2009, Stern et al. 2011). While the precise mechanism leading to ALS is not completely clear, elevated glutamate concentrations may be involved in the process by elevation of D-serine and directly activating NMDA receptors (Paul and de Belleruche 2012). Recently, evidence has

Table IV

Mean arterial blood pressure (MABP) and heart rate (HR), measured at different time points throughout the exercise session									
	0	30	60	90	120	150	180	210	240
MABP	93 ± 3	111 ± 6*	107 ± 3*	86 ± 3**	82 ± 2**	84 ± 2**	85 ± 3**	83 ± 2**	86 ± 2*
HR	71 ± 3	168 ± 4**	163 ± 6**	88 ± 3**	81 ± 3**	77 ± 3*	76 ± 3	74 ± 4	78 ± 4*

Mean arterial blood pressure increased transiently, peaking at 30 minutes (**P*<0.01), and started to consistently decline at 90 minutes (***P*<0.001) after initiation of exercise, reaching lower than baseline levels. Heart rate was increased at 30, 90, 120 (***P*<0.001) and 150 (**P*<0.01) and returned to baseline levels at 180 minutes after the start of exercise. Heart rate was measured to be elevated again at 240 minutes (**P*<0.01). Data is presented as mean ± SEM.

be found demonstrating that the homeostasis of glutamate in brain fluids is not only due to intraparenchymal glutamate uptake but is also based on a naturally occurring efflux of excess glutamate from brain interstitial fluids into blood (Teichberg et al. 2009). This efflux can be significantly accelerated by the administration of blood glutamate scavengers such as oxaloacetate and pyruvate which, by decreasing blood glutamate levels, increase the driving force for the up-hill efflux of glutamate from brain fluids into blood (Gottlieb et al. 2003). Blood glutamate scavenging has further been shown to provide significant brain neuroprotection after closed head injury (Zlotnik et al. 2007, 2008, 2009, Leibowitz et al. 2012).

Recently we have found that stress causes a transient decrease in blood glutamate levels, affording neuroprotection (Zlotnik et al. 2010a). Stress after injury is known to activate adaptive responses allowing the body to marshal its forces to confront the threat and provide protection. One of these responses is the release of adrenaline. Brain injury is associated with a significant catecholamine surge and adrenaline levels have been observed to increase several fold in patients with closed head injury (Tran et al. 2008). We have shown in naïve rats, that β -2 activation by adrenaline or administration of CRF, causes a significant reduction in blood glutamate concentration (Zlotnik et al. 2010a). The precise mechanisms by which these effectors affect blood glutamate levels are unclear, but their effects may be exerted at different sites including glutamate releasing or pumping organs such as the liver and skeletal muscles, or the activation or enhancement of naturally occurring scavenging mechanisms. Under normal conditions, blood glutamate levels are in a steady state regulated by a glutamate release from the liver and an uptake mainly in skeletal muscles (Hediger and Welbourne 1999). A stress-induced inhibition of the hepatic glutamate release or an activation of the glutamate transport into muscles, are both possible mechanisms for a decrease of blood glutamate levels.

Another proposed reaction to stress is the leakage of cytoplasmic enzymes into the blood by causing cell injury or increased cell membrane permeability. Various studies in animals and humans have found elevation in AST, ALT, CPK, LDH and circulating catecholamine levels, when subjected to stress (Meltzer 1971, Sen et al. 1992, Apple et al. 1993, Kayashima et al. 1995). Other studies have found that the enzyme elevation and catecholamine surge following stress is

suppressed in animals pretreated with β receptor antagonists (Arakawa et al. 1997). This is in accord with our previous findings demonstrating the effect of β receptor antagonists on the naturally occurring decrease of blood glutamate levels and its influence on neurological outcome in rats subjected to stress and TBI (Zlotnik et al. 2010a, 2011). Basing on the findings listed above one would expect that a catecholamine surge as a result of physical exercise should produce a decrease in blood glutamate. We did not observe such a reduction. This finding may be easily explained by the simultaneous large glutamate release from skeletal muscle and its shift into the bloodstream, which may mask any subtle glutamate reducing effects of catecholamines. Veloergometry and anaerobic metabolism are expected to cause muscle damage and a subsequent leak of cytoplasmic enzymes and amino acids into the circulating bloodstream. Previously in a rat model, we found a rapid distribution (minutes) of radiolabeled glutamate into peripheral tissues, with skeletal muscle serving as the major depot (59% of excess glutamate) (Klin et al. 2010). A reverse process of muscle damage is expected to release glutamate from muscle into the blood and cause an elevation in blood glutamate levels. A similar finding was observed in a rat model of hyperthermia, whereby skeletal muscle damage was accompanied by significant leak of glutamate from the skeletal muscle, leading to significant elevations in blood glutamate concentrations (Zlotnik et al. 2010b). Elevations in blood glutamate levels may decrease the brain-to-blood glutamate driving force, thus affecting neurological outcome when TBI is accompanied by skeletal-muscular trauma. Studies in athletes and laymen submitted to extreme physical exercise found a pattern of initial elevation of glutamate and alanine followed by a decrease in blood levels throughout the recovery process (Blomstrand et al. 1988, Newsholme and Blomstrand 1996, Yan et al. 2009).

In Blomstrand's study on human subjects performing leg-press exercises, glutamate and amino acids were assessed in plasma and muscle, before, during and after exercise (Blomstrand and Essen-Gustavsson 2009). Plasma glutamate did not reach a statistically significantly elevated level during exercise, a significant buildup of alanine, marking the degradation of glutamate, was found in plasma. In the recovery period, a rapid significant decrease in glutamate was evident, as demonstrated in our study. The discrepancy between these two studies may be explained by the

difference in the exercise protocol, which was much more intense in our study. These findings imply that the levels of stress and muscle damage have a major impact on the elevation of blood glutamate and that sympathetically mediated mechanisms have a capacity of rapidly decreasing blood glutamate levels, which is limited by the amount of glutamate released into circulating blood. Furthermore, Blomstrand and Essen-Gustavsson (2009) found a reduction in muscle glutamate concentrations following exercise, indicating a significant depletion of glutamate from within muscle due to physical exercise. Findings in our study are in agreement with the described pattern of glutamate: an initial increase and subsequent rapid decrease as a reaction to extreme physical exercise. Glutamate was elevated within 30 minutes until the end of the exercise, thereafter rapidly decreasing towards baseline in the 3 hours following exercise. The rapid decline in blood glutamate implies the existence of an autoregulatory mechanism of glutamate uptake and metabolism, maintaining blood glutamate levels within a normal range.

AST and ALT are blood resident enzymes, which in conjunction with their co-substrates oxaloacetate and pyruvate, respectively, convert glutamate into 2-ketoglutarate. Therefore, accumulation and elevation of 2-ketoglutarate together with elevated AST and ALT levels accompanying or following the elevation of glutamate indicates the activation of AST-oxaloacetate or ALT-pyruvate pathway. Along the course of exercise we found a gradual build-up of 2-ketoglutarate, peaking at 240 minutes with a two-fold concentration ($t_0 = 402 \pm 44 \mu\text{M/l}$, $t_{240} = 815 \pm 59 \mu\text{M/L}$, $P < 0.001$), indicating the continuous metabolism of excess glutamate. The initial elevation of glutamate, but normal-decreased levels at 90 minutes, together with the buildup of 2-ketoglutarate, indicates that the initial rapid elevation of glutamate exceeds the elimination capacity. However, following the initial surge, glutamate breakdown continues in a rate capable of converting and eliminating excess glutamate leaking from injured muscle fibers. As mentioned above, the build-up of 2-ketoglutarate requires increased activity of AST and ALT. We found a significant increase in AST and ALT by 60 minutes after exercise initiation following a gradual decline. AST levels remained elevated at 240 minutes. This finding is in accord with previous studies demonstrating an elevation in these enzymes, follow-

ing different types of stress (Kayashima et al. 1995, Arakawa et al. 1997). Nevertheless, the elevation in transaminases may be explained also by exertional myocyte damage or other physiologic factors such as moderate dehydration or redistribution into interstitial spaces. This study could not distinguish the actual reason for this rise, but the close association to glutamate rise, and the variety of situations other than exercise in which this rise is seen, support this as an adaptive response to glutamate. Blocking these pathways, thus causing the accumulation of glutamate, has been previously shown to deteriorate neurological outcomes following TBI, likely by influencing the brain-to-blood glutamate gradient (Zlotnik et al. 2009). Skeletal muscle and splanchnic organs are rich with glutamate (Marliss et al. 1971) and the mechanical injury of cell membranes may cause glutamate to leak down its concentrations gradient, from muscle cells into the blood. The strong increases in blood levels of CPK and myoglobin as the exercise protocol progressed, demonstrating injury to the muscle membranes, were further associated with increases in glutamate levels in the blood. This may be of importance in clinical scenarios such as multi-trauma, crush injuries, compartment syndrome, severe hyperthermia, heat stroke and rhabdomyolysis where extensive muscle damage may accompany brain injury.

In this study we used serum lactate, myoglobin and CPK which were significantly elevated during the exercise and throughout the recovery period (at least 240 minutes) as markers of anaerobic metabolism and muscle damage. Myoglobin and CPK are known to increase in intense exercise due to muscle damage (Meltzer 1971, Roti et al. 1981, Ross et al. 1983). This in turn leads to a significant release of glutamate from its major depot, causing an elevation in blood glutamate. Based on findings in this study and previous studies, we suggest that an important mechanism involved in glutamate regulation is the glutamate – 2-ketoglutarate pathway.

Values of blood pressure and heart rate were significantly elevated during the exercise period. The simultaneous increase in heart rate and mean arterial blood pressure reflects an increase in cardiac output, indicating the intensity of exercise as well as activation of the sympathetic nervous system and a release of catecholamines. Vasodilatation due to exercise, with the rapid decrease in heart rate after cessation of exer-

cise, may explain the decrease in mean arterial blood pressure that was also seen. Core body temperature remained unchanged throughout the experiment. The increase of heat production was most likely countered by the body's compensatory mechanisms such as sweating and vasodilatation.

In a recent study on a rat model of hyperthermia, rats were subjected to different levels of heat stress (Zlotnik et al. 2010b). The results of this study demonstrated that mild hyperthermia decreases blood glutamate levels, presumably due to sympathetic activation. Treatment with propranolol prior to increasing the rats' body temperature prevented this effect and precluded the hyperthermia-induced reduction of blood glutamate at 38°C and 39°C. Severe hyperthermia led to an increase in blood glutamate concentrations which could not be prevented by treatment with the blood glutamate scavenger oxaloacetate or pretreatment with the non-selective β -adrenergic receptor blocker propranolol.

Some studies find that exercise has beneficial effects on brain function, by increasing expression of genes involved in brain function (Tong et al. 2001, Cotman and Berchtold 2002, O'Callaghan et al. 2007), and enhancing proliferation of neuronal stem cells and proliferation factors (Wu et al. 2008). In our study, we did not look at markers of brain function, but the increase of blood glutamate during exercise may imply opposite results. Nevertheless, the transient nature of this rise, and the rapid elimination of blood glutamate after exercise may be part of a complex regulatory mechanism focused at maintaining normal brain function, offsetting the effects of increased glutamate.

One limitation of this study is the absence of data regarding glutamate levels in muscle tissue. It would be expected that when focusing on exercise and muscle, such an evaluation should be performed. Though this would have been interesting, it is out of the scope of this study. Since it has already been established that muscle is a major glutamate depot, we were primarily interested in the uptake and release of glutamate in and out of the blood, and the compartment in which circulating glutamate is presumed to have an impact on brain glutamate concentrations. Another limitation is due to the fact that the original fluorimetric analysis of glutamate done by Graham and Aprison (1966) was performed on nerve tissue. Aside from

studies done by our group (Teichberg et al. 2009, Klin et al. 2010, Zlotnik et al. 2011, Boyko et al. 2012), several other studies have used fluorimetric analysis for determination of blood/plasma glutamate levels (Gottlieb et al. 2003, Mitani et al. 2006). Ferrarese and coworkers (2001) describe a similar method for determination of increased glutamate in CSF and plasma of patients with HIV dementia. This method has been challenged by Graham and Aprison (1966) stating the possible artifacts which may possibly cause a wrong determination of plasma and CSF glutamate (Epsey et al. 2002). While this issue remains debatable, our study does not intend to determine the absolute levels of normal and abnormal blood glutamate. We are rather interested in the changes in blood glutamate and their association with different physiological mechanisms. Analysis was done using repeated measures ANOVA, so any inaccuracy in the measurement should be consistent, thus allowing us to arrive at similar conclusions. Nutritional status could influence the glutamate metabolism (Resende et al. 2011), thus despite the similar diet consumed by the study subjects, some variability may be attributed to different nutritional status.

CONCLUSIONS

Insight into the physiology and regulation of glutamate levels in the blood during stress and muscle damage may facilitate new efforts to decrease the elevated glutamate levels in the brain during acute brain events, such as TBI and stroke. The blood glutamate scavengers, oxaloacetate and pyruvate, have shown great success in decreasing the blood glutamate levels following CHI, thereby preventing secondary brain injury under normothermic conditions (Gottlieb et al. 2003, Zlotnik et al. 2007, 2008). Other treatment modalities, targeted on prevention of the release of glutamate from muscle tissue, or enhancement of uptake mechanisms, may prove to be beneficial as well. Future studies should further examine the effects of the increased release of glutamate from muscle and its consequent elevation in blood, as well as whether induced increases in blood glutamate concentrations may be used to effectively treat muscle damage.

REFERENCES

- Apple JK, Minton JE, Parsons KM, Unruh JA (1993) Influence of repeated restraint and isolation stress and electrolyte administration on pituitary-adrenal secretions, electrolytes, and other blood constituents of sheep. *J Anim Sci* 71: 71–77.
- Arakawa H, Kodama H, Matsuoka N, Yamaguchi I (1997) Stress increases plasma enzyme activity in rats: differential effects of adrenergic and cholinergic blockades. *J Pharmacol Exp Ther* 280: 1296–1303.
- Baker AJ, Moulton RJ, MacMillan VH, Shedden PM (1993) Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans. *J Neurosurg* 79: 369–372.
- Berl S, Lajtha A, Waelsch H (1961) Amino acid and protein metabolism of the brain. VI. Cerebral compartments of glutamic acid metabolism. *J Neurochem* 7: 186–197.
- Blomstrand E, Celsing F, Newsholme EA (1988) Changes in plasma concentrations of aromatic and branched-chain amino acids during sustained exercise in man and their possible role in fatigue. *Acta Physiol Scand* 133: 115–121.
- Blomstrand E, Essen-Gustavsson B (2009) Changes in amino acid concentration in plasma and type I and type II fibres during resistance exercise and recovery in human subjects. *Amino Acids* 37: 629–636.
- Boyko M, Stepensky D, Gruenbaum BF, Gruenbaum SE, Melamed I, Ohayon S, Glazer M, Shapira Y, Zlotnik A (2012) Pharmacokinetics of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase and their blood glutamate-lowering activity in naive rats. *Neurochem Res* 37: 2198–2205.
- Bullock R, Zauner A, Woodward JJ, Myseros J, Choi SC, Ward JD, Marmarou A, Young HF (1998) Factors affecting excitatory amino acid release following severe human head injury. *J Neurosurg* 89: 507–518.
- Camacho A, Massieu L (2006) Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. *Arch Med Res* 37: 11–18.
- Castillo J, Davalos A, Alvarez-Sabin J, Pumar JM, Leira R, Silva Y, Montaner J, Kase CS (2002) Molecular signatures of brain injury after intracerebral hemorrhage. *Neurology* 58: 624–629.
- Castillo J, Davalos A, Naveiro J, Noya M (1996) Neuroexcitatory amino acids and their relation to infarct size and neurological deficit in ischemic stroke. *Stroke* 27: 1060–1065.
- Castillo J, Davalos A, Noya M (1997) Progression of ischaemic stroke and excitotoxic aminoacids. *Lancet* 349: 79–83.
- Chio A, Calvo A, Dossena M, Ghiglione P, Mutani R, Mora G (2009) ALS in Italian professional soccer players: the risk is still present and could be soccer-specific. Amyotrophic lateral sclerosis: official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases 10: 205–209.
- Cohen-Kashi-Malina K, Cooper I, Teichberg VI (2012) Mechanisms of glutamate efflux at the blood-brain barrier: involvement of glial cells. *J Cereb Blood Flow Metab* 32: 177–189.
- Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends in neurosciences* 25: 295–301.
- Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65: 1–105.
- Davalos A, Castillo J, Serena J, Noya M (1997) Duration of glutamate release after acute ischemic stroke. *Stroke* 28: 708–710.
- Espey MG, Basile AS, Heaton RK, Ellis RJ (2002) Increased glutamate in CSF and plasma of patients with HIV dementia. *Neurology* 58: 1439. (author reply 1439–1440)
- Fu FH, You CY, Kong ZW (2002) Acute changes in selected serum enzyme and metabolite concentrations in 12- to 14-yr-old athletes after an all-out 100-m swimming sprint. *Percept Mot Skills* 95: 1171–1178.
- Gottlieb M, Wang Y, Teichberg VI (2003) Blood-mediated scavenging of cerebrospinal fluid glutamate. *J Neurochem* 87: 119–126.
- Graham LT, Jr., Aprison MH (1966) Fluorometric determination of aspartate, glutamate, and gamma-aminobutyrate in nerve tissue using enzymic methods. *Anal Biochem* 15: 487–497.
- Hammarqvist F, Ejesson B, Wernerman J (2001) Stress hormones initiate prolonged changes in the muscle amino acid pattern. *Clin Physiol* 21: 44–50.
- Hediger MA, Welbourne TC (1999) Introduction: glutamate transport, metabolism, and physiological responses. *Am J Physiol* 277: F477–480.
- Helms HC, Madelung R, Waagepetersen HS, Nielsen CU, Brodin B (2012) In vitro evidence for the brain glutamate efflux hypothesis: brain endothelial cells cocultured with astrocytes display a polarized brain-to-blood transport of glutamate. *Glia* 60: 882–893.
- Johnston MV, Trescher WH, Ishida A, Nakajima W (2001) Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatr Res* 49: 735–741.

- Kayashima S, Ohno H, Fujioka T, Taniguchi N, Nagata N (1995) Leucocytosis as a marker of organ damage induced by chronic strenuous physical exercise. *Eur J Appl Physiol Occup Physiol* 70: 413–420.
- Klin Y, Zlotnik A, Boyko M, Ohayon S, Shapira Y, Teichberg VI (2010) Distribution of radiolabeled l-glutamate and d-aspartate from blood into peripheral tissues in naive rats: significance for brain neuroprotection. *Biochem Biophys Res Commun* 399: 694–698.
- Koura SS, Doppenberg EM, Marmarou A, Choi S, Young HF, Bullock R (1998) Relationship between excitatory amino acid release and outcome after severe human head injury. *Acta Neurochir Suppl* 71: 244–246.
- Kratz A, Lewandrowski KB, Siegel AJ, Chun KY, Flood JG, Van Cott EM, Lee-Lewandrowski E (2002) Effect of marathon running on hematologic and biochemical laboratory parameters, including cardiac markers. *American journal of clinical pathology* 118: 856–863.
- Leibowitz A, Boyko M, Shapira Y, Zlotnik A (2012) Blood glutamate scavenging: insight into neuroprotection. *International journal of molecular sciences* 13: 10041–10066.
- Marliss EB, Aoki TT, Pozefsky T, Most AS, Cahill GF, Jr. (1971) Muscle and splanchnic glutamine and glutamate metabolism in postabsorptive and starved man. *J Clin Invest* 50: 814–817.
- Meltzer HY (1971) Plasma creatine phosphokinase activity, hypothermia, and stress. *Am J Physiol* 221: 896–901.
- Mitani H, Shirayama Y, Yamada T, Maeda K, Ashby CR Jr., Kawahara R (2006) Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog Neuropsychopharmacol Biol Psychiatry* 30: 1155–1158.
- Mukherjee K, Ghosh NC (1987) Glutamic oxalacetic and glutamic pyruvic transaminase activities in different tissues of rats and guinea pigs exposed to varying levels of altitude stress for different periods of time. *Aviat Space Environ Med* 58(1): 18–23.
- Newsholme EA, Blomstrand E (1996) The plasma level of some amino acids and physical and mental fatigue. *Experientia* 52: 413–415.
- O'Callaghan RM, Ohle R, Kelly AM (2007) The effects of forced exercise on hippocampal plasticity in the rat: A comparison of LTP, spatial- and non-spatial learning. *Behavioural brain research* 176: 362–366.
- O'Kane RL, Martinez-Lopez I, DeJoseph MR, Vina JR, Hawkins RA (1999) Na(+)-dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood-brain barrier. A mechanism for glutamate removal. *J Biol Chem* 274: 31891–31895.
- Palmer AM, Marion DW, Botscheller ML, Swedlow PE, Styren SD, DeKosky ST (1993) Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model. *J Neurochem* 61: 2015–2024.
- Paul P, de Belleruche J (2012) The role of D-amino acids in amyotrophic lateral sclerosis pathogenesis: a review. *Amino Acids* 43: 1823–1831.
- Resende NM, de Magalhaes Neto AM, Bachini F, de Castro LE, Bassini A, Cameron LC (2011) Metabolic changes during a field experiment in a world-class windsurfing athlete: a trial with multivariate analyses. *Omics : a journal of integrative biology* 15: 695–704.
- Richards DA, Tolia CM, Sgouros S, Bowery NG (2003) Extracellular glutamine to glutamate ratio may predict outcome in the injured brain: a clinical microdialysis study in children. *Pharmacol Res* 48: 101–109.
- Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, Kochanek PM, Graham SH (2002) Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. *Brain Res* 943: 15–22.
- Ross JH, Attwood EC, Atkin GE, Villar RN (1983) A study on the effects of severe repetitive exercise on serum myoglobin, creatine kinase, transaminases and lactate dehydrogenase. *Q J Med* 52: 268–279.
- Roti S, Iori E, Guiducci U, Emanuele R, Robuschi G, Bandini P, Gnudi A, Roti E (1981) Serum concentrations of myoglobin, creatine phosphokinase and lactic dehydrogenase after exercise in trained and untrained athletes. *J Sports Med Phys Fitness* 21: 113–118.
- Sen CK, Marin E, Kretzschmar M, Hanninen O (1992) Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J Appl Physiol* 73: 1265–1272.
- Sen P, Mediratta PK, Ray A (1992) Effects of *Azadirachta indica* A Juss on some biochemical, immunological and visceral parameters in normal and stressed rats. *Indian J Exp Biol* 30: 1170–1175.
- Serena J, Leira R, Castillo J, Pumar JM, Castellanos M, Davalos A (2001) Neurological deterioration in acute lacunar infarctions: the role of excitatory and inhibitory neurotransmitters. *Stroke* 32: 1154–1161.
- Stern RA, Riley DO, Daneshvar DH, Nowinski CJ, Cantu RC, McKee AC (2011) Long-term consequences of repetitive brain trauma: chronic traumatic encephalopathy. *PM & R: The Journal of Injury, Function, and Rehabilitation* 3: S460–467.
- Sutedja NA, Fischer K, Veldink JH, van der Heijden GJ, Kromhout H, Heederik D, Huisman MH, Wokke JJ, van

- den Berg LH (2009) What we truly know about occupation as a risk factor for ALS: a critical and systematic review. *Amyotrophic Lateral Sclerosis: official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 10: 295–301.
- Teichberg VI, Cohen-Kashi-Malina K, Cooper I, Zlotnik A (2009) Homeostasis of glutamate in brain fluids: an accelerated brain-to-blood efflux of excess glutamate is produced by blood glutamate scavenging and offers protection from neuropathologies. *Neuroscience* 158: 301–308.
- Tong L, Shen H, Perreau VM, Balazs R, Cotman CW (2001) Effects of exercise on gene-expression profile in the rat hippocampus. *Neurobiol Dis* 8: 1046–1056.
- Tran TY, Dunne IE, German JW (2008) Beta blockers exposure and traumatic brain injury: a literature review. *Neurosurg Focus* 25: E8.
- Vesce S, Rossi D, Brambilla L, Volterra A (2007) Glutamate release from astrocytes in physiological conditions and in neurodegenerative disorders characterized by neuroinflammation. *Int Rev Neurobiol* 82: 57–71.
- Wu CW, Chang YT, Yu L, Chen HI, Jen CJ, Wu SY, Lo CP, Kuo YM (2008) Exercise enhances the proliferation of neural stem cells and neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice. *J Appl Physiol* 105: 1585–1594.
- Yamamoto T, Rossi S, Stiefel M, Doppenberg E, Zauner A, Bullock R, Marmarou A (1999) CSF and ECF glutamate concentrations in head injured patients. *Acta Neurochir Suppl* 75: 17–19.
- Yan B, A J, Wang G, Lu H, Huang X, Liu Y, Zha W, Hao H, Zhang Y, Liu L, Gu S, Huang Q, Zheng Y, Sun J (2009) Metabolomic investigation into variation of endogenous metabolites in professional athletes subject to strength-endurance training. *J Appl Physiol* 106: 531–538.
- Zauner A, Bullock R, Kuta AJ, Woodward J, Young HF (1996) Glutamate release and cerebral blood flow after severe human head injury. *Acta Neurochir Suppl* 67: 40–44.
- Zhang H, Zhang X, Zhang T, Chen L (2001) Excitatory amino acids in cerebrospinal fluid of patients with acute head injuries. *Clin Chem* 47: 1458–1462.
- Zlotnik A, Gurevich B, Tkachov S, Maoz I, Shapira Y, Teichberg VI (2007) Brain neuroprotection by scavenging blood glutamate. *Exp Neurol* 203: 213–220.
- Zlotnik A, Gurevich B, Cherniavsky E, Tkachov S, Matuzani-Ruban A, Leon A, Shapira Y, Teichberg VI (2008) The contribution of the blood glutamate scavenging activity of pyruvate to its neuroprotective properties in a rat model of closed head injury. *Neurochem Res* 33: 1044–1050.
- Zlotnik A, Gruenbaum SE, Artru AA, Rozet I, Dubilet M, Tkachov S, Brotfain E, Klin Y, Shapira Y, Teichberg VI (2009) The neuroprotective effects of oxaloacetate in closed head injury in rats is mediated by its blood glutamate scavenging activity: evidence from the use of maleate. *J Neurosurg Anesthesiol* 21: 235–241.
- Zlotnik A, Klin Y, Kotz R, Dubilet M, Boyko M, Ohayon S, Shapira Y, Teichberg VI (2010a) Regulation of blood L-glutamate levels by stress as a possible brain defense mechanism. *Exp Neurol* 224: 465–471.
- Zlotnik A, Gurevich B, Artru AA, Gruenbaum SE, Dubilet M, Leibowitz A, Shaked G, Ohayon S, Shapira Y, Teichberg VI (2010b) The effect of hyperthermia on blood glutamate levels. *Anesth Analg* 111: 1497–1504.
- Zlotnik A, Klin Y, Gruenbaum BF, Gruenbaum SE, Ohayon S, Boyko M, Sheiner E, Aricha-Tamir B, Shapira Y, Teichberg VI (2011) The activation of beta2-adrenergic receptors in naive rats causes a reduction of blood glutamate levels: relevance to stress and neuroprotection. *Neurochem Res* 36: 732–738.
- Zlotnik A, Leibowitz A, Gurevich B, Ohayon S, Boyko M, Klein M, Knyazer B, Shapira Y, Teichberg VI (2012a) Effect of estrogens on blood glutamate levels in relation to neurological outcome after TBI in male rats. *Intensive Care Med* 38: 137–144.
- Zlotnik A, Sinelnikov I, Dubilet M, Dubilet E, Leibowitz A, Ohayon S, Boyko M, Shapira Y, Teichberg V (2012b) Effect of glutamate and blood glutamate scavengers oxaloacetate and pyruvate on neurological outcome and pathohistology of the hippocampus after traumatic brain injury in rats. *Anesthesiology* 116: 73–83.