Exogenous pyruvate prevents stress-evoked suppression of mitogen-stimulated proliferation

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Abstract

Although the phenomenon that psychological stress influences disease onset and progression is well established, the mechanisms underlying stress-evoked compromise of immune function remain unspecified. To test the hypothesis that energetic shortages compromise immunity, we evaluated the effectiveness of pyruvate, a metabolic supplement, to prevent stress-evoked suppression of mitogen-stimulated splenocyte proliferation. Male C57BL/6 mice were subjected to 2 h of restraint once daily for 14 days. Consistent with previous studies, mitogen-stimulated splenocyte proliferation was reduced after restraint; in contrast, mice that received pyruvate injections immediately following each episode of restraint did not reduce splenocyte proliferation. In addition, restraint-evoked corticosterone elevation did not habituate in animals treated with pyruvate, suggesting that glucocorticoids are not exclusively immunosuppressive. The ratio of pyruvate to lactate, an index of aerobic metabolism, was elevated in mice exposed to restraint suggesting that mice exposed to restraint were preferentially using aerobic metabolism and producing more ATP per unit of pyruvate than non-restrained mice. Furthermore, two of the effective doses of pyruvate (0.5 and 500.0 mg/kg) altered glucose levels suggesting a metabolic function of the supplement. Although several different mechanisms could possibly mediate the changes in splenocyte proliferation, these results support the hypothesis that stress-evoked immunosuppression may be a function of metabolic energy shortages and can be prevented via pyruvate supplementation.

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1. Introduction

Disruption of homeostasis by a physical or perceived challenge (stressor) results in compensatory metabolic changes aimed to restore homeostasis (for review see McEwen and Wingfield, 2003; Sapolsky et al., 2000). Psychological stressors are major determinants of disease onset and progression (Balbin et al., 1999; Chrousos, 2000; Yang and Glaser, 2000); such stressors induce or intensify common human ailments including heart disease, cancer, irritable bowel syndrome, and depression (Goetz et al., 1998). An estimated one million US workers are absent on an average workday because of stress-related complaints, and direct medical expenses are nearly 50% higher for workers who report feeling stress (Goetz et al., 1998). Laboratory studies show that chronic exposure to stressors suppresses immune function in rats and mice (Dhabhar and McEwen, 1999; Tournier et al., 2001), and this suppression is associated with changes in glucocorticoid-mediated regulation of immune function (Bauer et al., 2001).

Although stress-evoked immunosuppression is well documented (i.e., Dhabhar, 2000; Gennaro et al., 1997; Glaser et al., 2000), relatively little is known about the mechanisms by which this suppression occurs. One hypothetical mechanism is that stress-evoked immunosuppression results from metabolic energy shortages. Exposure to repeated restraint is metabolically taxing and results in negative energy balance (Harris et al., 1998; Laugero and Moberg, 2000b; Laugero and...
Moberg, 2000c). In addition, mounting and maintaining an immune response is metabolically expensive (Demas et al., 1997; Laugero and Moberg, 2000a; Laugero and Moberg, 2000c; Martin et al., 2003), and limiting metabolic fuel can reduce immune function (Demas et al., 1997; Demas et al., 2003; Dreau et al., 1998; Giovambattista et al., 2000), suggesting that stress-evoked immunosuppression could be mediated by energy shortages.

A recent study supports the assertion that energy depletion by repeated exposure to stressors suppresses immune function (Drazen et al., 2001). Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that is activated by DNA strand breaks and participates in DNA repair. The normal function of PARP-1 requires the use of both NAD+ and ATP; if DNA damage is extensive, then PARP-1 becomes cytotoxic by exhausting cellular metabolic fuel (Ha and Snyder, 2000). PARP-1 appears to mediate stress-evoked immunosuppression. Immune function of PARP−/− mice was unaffected by chronic exposure to a stressor as compared to wild-type mice (Drazen et al., 2001). Because PARP-1 exhausts metabolic fuel and the absence of this enzyme suppresses the cytotoxic effects of chronic stress, immunosuppression may reflect stress-evoked depletion of metabolic fuel.

If reduced metabolic fuels mediate stress-evoked immunosuppression, then increasing metabolic fuel may be one mechanism for coping with depleted cellular energy and preserving energy balance during exposure to chronic stress. To this effect, food consumption increases after exposure to stressors in humans, with a preference for high caloric “sweet” foods (Epel et al., 2000). In addition, the hypothalamic–pituitary–adrenal (HPA) axis response to a stressor is attenuated in rats consuming a high caloric or high carbohydrate diet (Levin et al., 2000; Strack et al., 1997), and glucose ingestion blocks the behavioral impairment (learned helplessness) of rats exposed to inescapable shock (Minor and Saade, 1997). Furthermore, sucrose ingestion also stimulates food intake and storage and decreases basal adrenocorticotropic hormone (ACTH) concentrations in adrenalectomized rats, similar to the effect of systemic corticosterone replacement (Laugero, 2001). Taken together, these findings, and evidence indicating that intracerebroventricular (icv) corticosterone blocks the metabolic restoration mediated by sucrose ingestion (Laugero et al., 2002), suggest that the effects of both corticosterone and sucrose ingestion are exerted in the periphery, and support the hypothesis that the cytotoxic effects of exposure to chronic stress may be attenuated by compensation for energy shortages.

Although increasing sucrose may ameliorate stress-induced immunosuppression, prolonged increase in sugar consumption may have detrimental effects (for review see Hung et al., 2003). An alternative fuel source is pyruvate. Pyruvate is the natural byproduct of glycolysis and a required substrate for the formation of acetyl CoA, the entry point of the oxidative pathway. The end products of the oxidative pathway include both NAD+ and ATP; recall that these are the same energy sources depleted by PARP overactivation. Therefore, the supplementation of pyruvate, which bypasses the metabolically expensive process of glycolysis, may generate additional metabolic fuel, NAD+ and ATP, in a compromised system. Pyruvate has proven to be protective against damage induced by forebrain ischemia (Lee et al., 2001), zinc-induced neurotoxicity (Kawahara et al., 2002), and pancreatic islet cell death and diabetes (Chang et al., 2003). The protective function of pyruvate is in part attributable to a normalization of cellular levels of NAD+ (Sheline et al., 2000) and indirect evidence suggests that NAD+ is depleted during repeated stressors (Drazen et al., 2001). As an initial assessment of the immunoprotective capacity of pyruvate, we tested the prediction that daily supplementation with pyruvate would maintain mitogen-stimulated splenocyte proliferation following exposure to restraint stressors.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice (16 weeks) were housed individually in polycarbonate cages (28 × 17 × 12 cm) from the onset of the study in rooms programmed to be illuminated for 14 h/day (LD14:10, lights illuminated at 0000 h EST) and maintained at temperatures of 20 ± 4°C and relative humidity of 50 ± 5%. Tap water was available ad libitum throughout the study. At the start of the experiment mice were randomly assigned to one of 12 experimental groups: (1) restraint stress + saline, (2) restraint stress + 0.5 mg/kg pyruvate, (3) restraint stress + 5.0 mg/kg pyruvate, (4) restraint stress + 50.0 mg/kg pyruvate, (5) restraint stress + 500.0 mg/kg pyruvate, (6) saline, (7) 0.5 mg/kg pyruvate, (8) 5.0 mg/kg pyruvate, (9) 50.0 mg/kg pyruvate, (10) 500.0 mg/kg pyruvate, (11) handled, or (12) undisturbed. The experiment was further divided into two blocks such that half of the subjects from each of these groups was tested in each of the two runs of the experiment. This design allowed us to replicate our initial findings.

2.2. Feeding schedule

Food (LabDiet 5001; PMI Nutrition; Brentwood, MO) intake was monitored beginning 10 days prior to the start of the experiment and continued for the duration of the experiment for animals exposed to restraint stress + saline. During the experiment, food intake of the group that received daily restraint stress + saline was
used to determine the portion of food provisions allotted to the remaining groups. Basal food intake was 4.33 ± 0.06 g per day and intake was significantly lower on days 1, 3, and 9 of the experiment. Individual food allotments for mice in all other groups were calculated based on the daily change from basal intake of this group (n = 9).

2.3. Restraint stressor

Mice were placed in adequately ventilated clear polypropylene restrainers (50-ml conical tubes measuring 9.7 cm in length and internal diameter of 2.8 cm) for 2 h/day; breathing was monitored for all mice to make certain animals were not compressed. Animals were subjected to randomly timed (between 0700 and 1200 h) restraint for 2 weeks.

2.4. Pyruvate injections

Sodium pyruvate (0.5, 5.0, 50.0, and 500.0 mg/kg; Sigma Chemical, St. Louis, MO) dissolved in sterile water was given by intraperitoneal (i.p.) injection immediately following each episode of restraint (i.e., upon removal from the restraint tube). Control mice were injected i.p. with osmolarity-matched NaCl solution immediately following removal from the restraint tube. Groups not exposed to restraint stress received a sodium pyruvate (0, 0.5, 5.0, 50.0, and 500.0 mg/kg) injection at the same time of day as the animals exposed to restraint stress. Additionally, one group was handled each day and one group remained undisturbed for the duration of the experiment.

2.5. Splenocyte proliferation

Mitogen-induced proliferation was assessed on day 14 within 15 min of treatment. Spleens were aseptically removed, and splenocytes were assayed for proliferation in response to the mitogen concanavalin A (ConA). Splenocyte proliferation was assessed using a colorimetric assay based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS/PMS; Promega, Madison, WI). Splenocytes were separated from tissue by compressing the whole spleen between sterile frosted glass slides. The resulting slurry was suspended in 3 ml of RPMI-1640 (Sigma Chemical, St. Louis, MO) and layered onto 2 ml of Ficoll (Sigma Chemical, St. Louis, MO). Tubes were spun at 2500 rpm for 30 min and then the white blood cell layer was removed, placed in a sterile tube, and spun for 10 min at 1500 rpm. The resulting pellet was suspended in 200 μl of supplemented culture medium (RPMI-1640/Hepes supplemented with 1% penicillin (5000 U/ml)/streptomycin (5000 μg/ml), 1% glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5 × 10⁻² M/ml), and 10% heat-inactivated fetal bovine serum). Splenocyte counts and viability were determined with a hemacytometer and trypan blue exclusion. Viable cells were adjusted to 2 × 10⁶ cells/ml by dilution with culture medium, and 50 μl aliquots of each cell suspension (i.e., 100,000 cells) were added to the wells of sterile culture plates. Con A (Sigma Chemical, St. Louis, MO) was diluted with culture medium to concentrations of 10 μg/ml because it was previously determined that this concentration is optimal for stimulation of mouse splenocytes. Plates were incubated at 37°C with 5% CO₂ for 48 h prior to addition of 20 μl of MTS/PMS solution per well. Plates were then incubated at 37°C with 5% CO₂ for an additional 4 h. The OD of each well was determined with a microplate reader (490 nm; Bio-Rad, Benchmark). Mean OD values for each set of duplicates were used in subsequent statistical analyses.

2.6. RIA procedures

Corticosterone concentrations were measured from blood collected 5 days prior to experiment onset, and on days 1 and 14 to assess the effect of the stressor. Within 15 min after the termination of restraint on days 1 and 14, a blood sample was obtained from the retro-orbital sinus under light anesthesia and serum corticosterone assayed by radioimmunoassay (RIA). For control groups a blood sample was obtained 5 days prior to experimental onset and immediately following injection or handling on day 14. The RIA was conducted following the guidelines in the ICN (Costa Mesa, CA) 125I double-antibody kit instructions. The RIA is highly specific, cross-reacting at less than 1% with other hormones and a detection limit of 5 ng/ml. The coefficients of variation were <10%, and the intra-assay variation was <4%.

2.7. Metabolic assays

Pyruvate and lactate concentrations, as well as the pyruvate:lactate ratio were measured on day 14 to assess the effect of chronic stress on metabolic function. Because pyruvate is converted to lactate during anaerobic metabolism and this pathway produces fewer ATP per unit of pyruvate than the aerobic pathway, assessing the pyruvate:lactate ratio provides an index of metabolic efficiency. Within 15 min after restraint stress and injection or injection alone, trunk blood was collected for analysis of both pyruvate and lactate concentrations using enzymatic determination kits (Sigma Diagnostics, St. Louis, MO). Trunk blood collected on day 14 was also used to assess glucose concentrations using the FreeStyle blood glucose monitoring system (TheraSense, Alameda, CA).

2.8. Data analyses and statistics

Splenocyte proliferation in response to 10 μg of Con A is expressed as mean OD. Due to large variability
within groups, data points more than two standard deviations away from the mean were excluded from further analysis and the remaining data were log transformed. Pyruvate and lactate values are expressed as the percent of the plate positive control and then averaged within each group. Body masses for day 14 of the experiment are expressed as the percent of basal body mass for each animal and then averaged within each group. Glucose and corticosterone concentrations are averaged within each group. Statistical analyses for splenocyte proliferation, body mass, glucose concentrations, and pyruvate concentrations were conducted in three steps. First, data from control mice exposed to either a saline injection or daily handling were compared using two-tailed t tests. Because no difference was found for any of the measures, data from these groups were collapsed and used as the control for the remainder of the statistical analyses. Second, data from animals exposed to restraint stress and saline injection were compared to the control group using two-tailed t tests. Lastly, one-way ANOVAs were used to compare data from animals exposed to restraint stress and pyruvate injection and pyruvate injection alone to the control. In the case of lactate values all analyses were the same as described above with the exception of the comparison of data from animals exposed to pyruvate injections alone to the control animals. These data lacked a normal distribution and therefore a Kruskal–Wallis one-way analysis of ranks was conducted. Corticosterone concentrations were analyzed within groups as a function of day of stress exposure and drug condition by using a two factor repeated measures ANOVA with one factor repetition. Dunnett’s Method was used to further distinguish among groups, and all differences were considered statistically significant if p < .05.

3. Results

3.1. Splenocyte proliferation

Splenocyte proliferation in response to 10 μg Con A was differentially affected by exposure to 14 days of control treatments, restraint stress + saline injections and restraint stress + pyruvate injections (F(5, 53) = 3.478; p = .01; Fig. 1). Proliferation was suppressed in animals exposed to 14 days of restraint stress + saline injections (n = 6) compared to control animals (n = 16; t(20) = 2.379; p = .03). Proliferation was not suppressed in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; n = 8 per group). At the conclusion of 14 days of pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; n = 7, 8, 8, 7, respectively), splenocyte proliferation was unaltered as compared to control animals (n = 16).

![Fig. 1. In vitro splenocyte proliferation in response to 10 μg Con A. (A) Restraint (n = 6) suppressed proliferation as compared to control animals (n = 16). Pyruvate prevented restraint-induced suppression (n = 8 per group). (B) Pyruvate injections in the absence of restraint did not impact splenocyte proliferation (n = 7, 8, 8, 7, respectively). * p < .05 as compared to control; # p < .05 as compared to restraint + saline group.](image)

3.2. Corticosterone

Basal corticosterone concentrations did not differ among any of the experimental conditions tested. Furthermore, there were no differences in basal corticosterone concentrations between the mice in the experimental conditions and those observed in undisturbed control mice. Analysis of the corticosterone concentrations of animals exposed to restraint stress did not indicate a main effect of condition; however, there was a main effect of time (F(2, 122) = 112.74; p < .001; Fig. 2) and an interaction of condition and time (F(8, 122) = 3.08; p = .005). All animals exposed to restraint stress exhibited significantly elevated corticosterone concentrations immediately following the first exposure. This increase was attenuated following the fourteenth exposure in animals subject to restraint stress + saline injection (p < .05). Animals exposed to restraint stress + pyruvate injection (0.5, 5.0, 50.0, and 500.0 mg/kg) continued to exhibit elevated corticosterone concentrations on day 14 and these values were not significantly different from those documented after the first restraint. Analysis of corticosterone concentrations of animals exposed to either handling or pyruvate injection (0.0, 0.5, 5.0, 50.0, and 500.0 mg/kg) indicated...
an effect of time ($p < .001$) but no effect of condition and no interaction. Posthoc analysis verified that corticosterone concentrations were elevated following the 14th exposure to either handling or pyruvate injection (0.0, 0.5, 5.0, 50.0, and 500.0 mg/kg).

### 3.3. Pyruvate

Serum pyruvate was similar in animals exposed to 14 days of restraint stress + saline injections ($n = 9$) compared to control animals ($n = 16$). Pyruvate concentrations were also similar in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; $n = 8$ per group; Fig. 3) compared to control animals ($n = 16$). Fourteen days of pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; $n = 7, 8, 8, 7$, respectively) altered pyruvate concentrations as compared to control animals ($n = 16$; $F(4,45) = 3.93$; $p < 0.01$) and posthoc analysis attributed this difference to an elevated pyruvate concentration in animals that received 500.0 mg/kg each day.

### 3.4. Lactate

Serum lactate was significantly lower in animals exposed to 14 days of restraint stress + saline injections ($n = 9$) compared to control animals ($n = 16$; $t(23) = 2.82$; $p = .01$; Fig. 3). Lactate concentrations were also reduced in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; $n = 8$ per group) compared to control animals ($n = 16$; $F(4,47) = 2.74$; $p < .05$). This difference is attributable to the significantly lower concentration of lactate for animals exposed to restraint.

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**Fig. 2.** Mean corticosterone concentrations (±SEM) for (A) restrained or (B) injection only groups. *, Significantly different from baseline concentrations; #, significantly different from day 1 value, $p < .05$.

**Fig. 3.** Restraint and pyruvate supplementation did not induce changes in mean (±SEM; A) pyruvate, however changes were induced in (C) lactate, and (E) pyruvate:lactate ratio ($n = 8$ per group) as compared to control animals ($n = 16$). Pyruvate supplementation induced changes in mean (±SEM; B) pyruvate, but not (D) lactate, nor (F) pyruvate:lactate ratio ($n = 7, 8, 8, 7$, respectively) as compared to control animals ($n = 16$). *$p < .05$. 

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stress + 5.0 mg/kg pyruvate. Fourteen days of pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8 \) per group) did not alter lactate concentrations as compared to control animals \((n = 16)\).  

3.5. Pyruvate:lactate ratio  
The pyruvate:lactate ratio was elevated in animals exposed to 14 days of restraint stress + saline injections \((n = 9)\) compared to control animals \((n = 16; t(23) = -3.18; p < .01; \text{Fig. 3})\). Pyruvate:lactate ratios were similar in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8 \) per group) compared to control animals \((n = 16)\). Similarly, 14 days of pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 7, 8, 8, 7 \), respectively) did not alter the pyruvate:lactate ratio as compared to control animals \((n = 16)\).  

3.6. Glucose  
Glucose values were similar in animals exposed to 14 days of restraint stress + saline injections \((n = 8)\) compared to control animals \((n = 16)\). Serum glucose concentrations were altered in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8 \) per group) compared to control animals \((n = 16; F(4,47) = 3.82; p = .01; \text{Fig. 4})\). Posthoc analysis attributed this difference to elevated glucose concentrations in animals that received either 0.5 or 500.0 mg/kg of pyruvate each day. Fourteen days of pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8 \) per group) did not alter glucose concentrations as compared to control animals \((n = 16)\).  

3.7. Body mass  
Body mass was significantly lower in animals exposed to 14 days of restraint stress + saline injections \((n = 9)\) compared to control animals \((n = 16; t(23) = 5.15; p < .001; \text{Fig. 5})\). Body mass was also reduced in animals exposed to 14 days of restraint stress + pyruvate injections (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8 \) per group) compared to control animals \((n = 16; F(4,47) = 12.54; p < .001)\). Posthoc analysis indicated that all groups had significantly lower body mass than the control group. Fourteen days of pyruvate injections alone (0.5, 5.0, 50.0, and 500.0 mg/kg; \( n = 8, 8, 8, 7 \), respectively) did not alter body mass as compared to control animals \((n = 16)\).  

4. Discussion  
Mice given daily supplementation of pyruvate, aimed to prevent energy shortage, maintained mitogen-stimulated proliferation following exposure to restraint stressors, whereas non-supplemented mice that were restrained for two weeks displayed compromised proliferation. Randomly timed restraint stressors elevated corticosterone concentrations and the magnitude of this elevation began to subside by the 14th exposure. Pyruvate supplementation prevented this habituation of the corticosterone response following repeated restraint stressors. Pyruvate metabolism appeared to be altered in
animals exposed to restraint such that pyruvate was preferentially metabolized by the aerobic pathway, likely leading to increased energy production. Finally, an interaction between restraint and pyruvate supplementation may have altered glucose metabolism, and thereby account for the immunoprotective effects of pyruvate supplementation.

Daily supplementation with sodium pyruvate following each restraint session prevented compromise of mitogen-stimulated proliferation induced by two-weeks of restraint stressors. These results are, to our knowledge, the first to show mitigation of the detrimental effects of chronic stressors on the immune system via a metabolic supplement. Previous work has illustrated the protective capacity of pyruvate when administered immediately following forebrain ischemia (Lee et al., 2001), hemorrhagic shock (Mongan et al., 2001), and hydrogen peroxide-induced toxicity (Desagher et al., 1997).

The corticosterone response elicited by exposure to the restraint stressor began to attenuate by the 14th exposure; however, this habituation was absent in the pyruvate-supplemented groups. This finding is consistent with previous results that suggest elevated glucocorticoid concentrations are not necessarily immunosuppressive (Dhabhar, 2002; Fleshner et al., 2001; Sapolsky et al., 2000). Elevated glucocorticoids have been linked to immune cell death (Dhabhar and McEwen, 1999; Tournier et al., 2001), and have been suggested to act as the primary mediator of stress-evoked immunosuppression (Bauer et al., 2001). However, glucocorticoids induce apoptotic cell death, which is typically adaptive (for review see Marsden and Strasser, 2003) as compared to the more damaging necrotic death that occurs when energy is too far depleted to fuel either apoptosis or DNA repair (Eguchi et al., 1997; Leist et al., 1997). If pyruvate satisfies the metabolic demand signaled by increased glucocorticoids, then pyruvate may shorten the amount of time that corticosterone concentrations are elevated after each exposure to the stressor, thereby decreasing both corticosterone-mediated apoptotic death and generalized necrotic death. These data confirm and extend previous results that the immune system can function in the presence of increased glucocorticoids (Dhabhar, 2002; Fleshner et al., 2001; Sapolsky et al., 2000), and suggest that, given sufficient metabolic fuel, repeated restraint stressors do not necessarily compromise immune function.

Metabolism was altered by both pyruvate supplementation and restraint stress. The highest dose of pyruvate tested (500.0 mg/kg) effectively increased serum pyruvate levels as measured 15 min following injection, suggesting that pyruvate supplementation reached the blood stream. Because pyruvate is metabolized at a rapid rate, it is possible that any increase produced by the lower doses of pyruvate was metabolized by the time of measurement. The increased pyruvate:lactate ratio in animals exposed to restraint stress is attributable to a decreased production of lactate. As lactate production suggests the use of a less efficient metabolic pathway, it appears that the animals exposed to restraint stress are producing the maximal amount of energy possible from the available resources. Increased efficiency in pyruvate metabolism may account for the limitation of the effectiveness of pyruvate supplementation to those animals exposed to restraint stressors.

Glucose concentrations were elevated in animals exposed to restraint stressors and either 0.5 or 500.0 mg/kg pyruvate. All glucose concentrations observed were well within the normal physiological range of mice, suggesting a subtle change induced by pyruvate supplementation, as might be expected in normal metabolism (Harkness and Wagner, 1995). Because glucose and corticosterone often work in opposition to one another, elevated glucose may be sufficient to result in a more expedient return to basal corticosterone concentrations. It is possible that supplementing glucose into the system would have a similar immunoprotective effect, because glucose ameliorates some of the behavioral effects of repeated exposure to stressors (Levine, 2000; Minor and Saade, 1997; Strack et al., 1997). However, excessive delivery of glucose can exacerbate other disease conditions (for review see Hung et al., 2003); furthermore, the possibility remains that the metabolically expensive process of glycolysis might be bypassed during the energetic challenge induced by exposure to restraint stressors such that the glucose is stored instead of metabolized.

Body mass was significantly decreased in all animals exposed to restraint, including those receiving pyruvate supplementation. Previous work has illustrated that reducing body mass can compromise the immune system (Lord et al., 1998). The observation that animals receiving pyruvate supplementation reduced body mass without suppressing their immune system, as suggested by intact mitogen-stimulated proliferation, provides additional support that the pyruvate supplementation is effectively providing metabolic fuel to the system.

The U-shaped dose–response curve of pyruvate supplementation with the lowest (0.5 mg/kg) and highest (500.0 mg/kg) doses exerting the greatest effect on splenocyte proliferation and glucose levels was somewhat unexpected. Although the highest dose of pyruvate (500.0 mg/kg) tested appears to be a viable candidate for metabolic restoration, the lowest dose (0.5 mg/kg) seems to be a less likely candidate, because a 25 g mouse only receives 1.25 μg of sodium pyruvate. Because the 500.0 mg/kg dose is successful in altering levels of pyruvate and the 0.5 mg/kg dose is not, it is possible that only the 500.0 mg/kg dose is directly supplying exogenous energy to the system. However, both the 0.5 and 500.0 mg/kg doses increase glucose concentrations in the blood. This
suggestions that the 0.5 mg/kg dose of pyruvate may be working through an indirect mechanism to increase metabolic fuel in the system, perhaps as a peripheral metabolic signal (for review see Dallman et al., 2003).

An alternative possibility is that stress-evoked immunosuppression is the result of oxidative stress. Psychological stress can increase free-radical production (Irie et al., 2000; Irie et al., 2002), and pyruvate has antioxidant properties (Mallet et al., 2002; Mongan et al., 2002; Shea et al., 2002), potentially accounting for its immunoprotective capacity in the context of repeated restraint stressors. Although antioxidant treatment prevents restraint-induced immunosuppression in mice (Wakikawa et al., 1999), antioxidant treatment may not be a reliable means of immunoprotection (Gleeson et al., 2001). Regardless, if the immunoprotective effects of pyruvate were restricted to only antioxidant activities, then the alterations in glucose concentrations and the corticosterone response would not be expected, suggesting that in the present study the effects of pyruvate were mediated via a metabolic pathway.

In conclusion, pyruvate supplements preserve mitogen-stimulated proliferation of splenocytes during exposure to chronic restraint stressors despite elevated corticosterone concentrations, and may do so by altering glucose metabolism. Because cells in the ex vivo culture are exposed to equivalent energy conditions, it is possible that other non-metabolic processes may have been affected by pyruvate supplementation. Although we assert that restraint is changing (in vivo) the ability of cells to proliferate in response to a challenge once they are separated out of the organism, several different and not mutually-exclusive mechanisms could be responsible (e.g., changes in the proportions or ratios of cell types, induction of anergy, or disruptions in cell surface signaling properties, etc). Additional studies are necessary to determine whether pyruvate supplementation can prevent stress-induced changes of immune function in vivo. A strong test of the assertion that pyruvate acts as a metabolic supplement would be the demonstration that pyruvate supplementation ameliorates immunosuppression induced by food restriction. The ability of a metabolic supplement to prevent stress-evoked immunosuppression has potential conceptual and clinical significance.

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