Phenylbutazone blocks the cytokine response following a high-intensity incremental exercise challenge in horses

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Abstract
This study tested the hypothesis that phenylbutazone would block the exercise-induced increase in cytokine markers of inflammation in blood. Blood samples were obtained from unfit Standardbred mares (age 10 ± 4 years, ~ 500 kg) before and after three different trials (standing control (CON), n = 9; exercise with phenylbutazone (EX-bute), n = 9; and exercise with water, n = 9). Comparisons were made for data collected in three trials, one where each horse underwent an incremental exercise test (graded exercise test (GXT)) where they were administered water as a placebo, a GXT following phenylbutazone administration (2 g given orally 2 h before the GXT) or standing parallel control where they stood quietly in stalls. During the GXT, horses ran on a treadmill (1 m s⁻¹ increases each min until fatigue, 6% grade). Blood samples were obtained 30 min before exercise, immediately after exercise and at 0.5, 1, 2, 4 and 24 h post-GXT or at matched time points during the parallel control trials. Samples were analysed using real-time PCR for measurement of mRNA expression of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin (IL)-6 in samples collected during all three trials, and for IL-1 and IL-10 in samples collected for the CON and EX-bute trials. Data were analysed using ANOVA for repeated measures, and where appropriate, post hoc separation of means utilized the Student–Newman–Keuls test. The null hypothesis was rejected when P < 0.05. There were no changes (P > 0.05) in IL-1, IL-6, IFN-γ or TNF-α during CON or following phenylbutazone administration. During the water trial, exercise resulted in significant increases in IFN-γ, IL-1 and TNF-α. It was concluded that high-intensity exercise results in a transient increase in the expression of inflammatory cytokines in blood that is blocked when phenylbutazone is administered to horses.

Keywords: horse; cytokines; non-steroidal anti-inflammatory drugs; exercise; equine; muscle

Introduction
The cytokines are low-molecular weight proteins that are produced by a variety of cells and tissues. They are released locally when cells are perturbed by a variety of challenges ranging from minor to severe. In the case of the latter, they play a key role in the modulation of inflammation and as a reaction to an infection or tissue injury. The cytokine response is complex and multifaceted, utilizing endocrine, paracrine and autocrine actions to facilitate intracellular communications. The resulting interactions between various
tissues, cells and signalling pathways can recruit many immune cell types, such as monocytes, lymphocytes and neutrophils to aid in the repair process to tissue injury\textsuperscript{1–6}. Cytokines can also modulate the inflammatory and immune response by exerting inhibitory effects on immune cells, resulting in restrictions on growth, differentiation and function\textsuperscript{1–6}. Prior studies of horses have reported that a variety of perturbations, such as obesity, ageing and exercise, cause increases in the expression of mRNA for interferon-\(\gamma\) (IFN-\(\gamma\)), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin (IL)-6 and a number of other cytokines\textsuperscript{7–13}.

TNF-\(\alpha\) is an acute pro-inflammatory cytokine that has been used as a general marker of inflammation\textsuperscript{1–6}. TNF-\(\alpha\) plays a role in the response to muscle damage, muscle proteolysis and impaired skeletal muscle glucose uptake\textsuperscript{1–6,14}. IFN-\(\gamma\) is a major pro-inflammatory cytokine synthesized by T-helper 1 and natural killer (NK) cells\textsuperscript{15}. It acts on stimulated macrophages, Tcytotoxic cells, NK cells and B lymphocytes, and is involved in nitric oxide synthesis, antibody production and antiviral activity\textsuperscript{15,16}. IL-6 has been documented to function in the multifacet cascade of events involved in the onset and modulation of inflammation\textsuperscript{1,2}. In that role, there are conflicting studies suggesting that IL-6 has both anti-inflammatory and pro-inflammatory roles\textsuperscript{1–6}. However, when it comes to its biological role during exercise, the preponderance of information on the effect of IL-6 demonstrates that it is an important signalling protein in the integrated control of glucose homeostasis in muscle\textsuperscript{1–6,18–21}. This role that IL-6 plays in glucose homeostasis has led researchers to classify IL-6 as a ‘myokine’ or a muscle cytokine\textsuperscript{2,14}.

The role of the cytokines in the modulation of the inflammatory response to exercise has been well documented in a large number of basic and applied research papers\textsuperscript{1–21}. On an applied level, many of those experiments have had the goal of understanding the mechanisms behind exercise-induced increases in the mediators of the inflammatory response. Even with a greater understanding of the mechanism behind the inflammatory response, the elusive objective of many studies has been to discern the fine line between minor muscle inflammation that evokes an adaptive response versus an excessive increase in markers of inflammation associated with more frank muscle damage, loss of function and performance\textsuperscript{1–21}. However, it should be noted that the cytokines not only play a role in inflammation, they also play an important role in pathways impacting multiple physiological responses to exercise\textsuperscript{1,2}. These pathways include those affecting a variety of neuroendocrine mechanisms associated with the control of peripheral and central mechanisms affecting exercise performance\textsuperscript{1,2}. Peripherally, the cytokines interact in the complex control of peripheral blood flow as well as

metabolic pathways used to fuel exercise\textsuperscript{1,2}. Centrally, they appear to have a role in the control of cardiovascular and respiratory function\textsuperscript{1,2}. The widespread nature of the role the cytokines play in the response to exercise suggests that their moderate increase, evoked by acute exercise, is appropriate and part of the protective mechanism dealing with the physiological challenge of exertion\textsuperscript{1,2}. Excessive exercise intensity, duration and resistance can cause significant increases in cytokines, resulting in post-exercise muscle soreness with a spectrum of symptoms that have been classified as delayed onset muscle soreness (DOMS)\textsuperscript{1,2}. In its extreme, DOMS can result in the classic signs of inflammation including heat, pain, swelling and loss of function.

These ergolytic effects of post-exercise inflammation can be countered through the use of non-steroidal anti-inflammatory drugs (NSAID), including various pharmaceutical and nutraceutical anti-inflammatory countermeasures\textsuperscript{12,15,22}. Recent studies of humans and horses have examined the administration of pharmaceutical and nutraceutical substances (food extracts having anti-inflammatory properties) to block the inflammatory response to very intense exertion\textsuperscript{12,13}. Among those pharmaceutical interventions, there is a long list of NSAIDs commonly given to both humans and horses\textsuperscript{22,23}. In the case of horses, the most utilized NSAID is phenylbutazone\textsuperscript{22}. Phenylbutazone is a very potent analgesic and anti-inflammatory agent\textsuperscript{22}. However, its systemic effects can also alter the normal exercise-induced response of the endocrine, cardiovascular and respiratory systems\textsuperscript{22–34}. Interestingly, no study to date has examined the effect of phenylbutazone on the cytokine response to exertion in the horse. Therefore, this study tested the hypothesis that phenylbutazone would alter the exercise-induced increase in cytokines measured in blood.

**Materials and methods**

**Animals and dosing**

The Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment. Nine healthy, unfit Standardbred mares (aged 10 ± 4 years, ~500 kg) were utilized for three different trials (standing control (CON, \(n = 9\)); exercise with phenylbutazone (EX-bute, 2 g given orally 2 h before the graded exercise test (GXT), \(n = 9\)); and exercise with water (EX-water, \(n = 9\)). The EX-water GXTs were conducted as part of a separate trial\textsuperscript{12} that was conducted months before the subsequent two rounds. The CON and EX-bute GXTs utilized seven of the nine horses used in the original nutraceutical experiment, with the addition of two mares that had been held in reserve and were housed and cared for, even though they were part of
the group used in the original experiment. All mares were accustomed to the laboratory and running on the treadmill before the start of the experiment.

During the study, the horses were housed as a group on a dry lot. Each mare was fed approximately 2.5 kg of total mixed ration hay cubes (Eckenberg Farms, Mattawa, WA, USA) twice a day (~5 kg·d⁻¹ total) at about 07.00 and 15.00 hours. Water and grass hay were provided ad libitum. The ration was formulated to meet the energy guidelines of the National Research Council (1989) for adult horses conducting periodic exercise. Mares were not fed on the morning of the exercise test.

**Graded exercise test**

The GXT used previously published methods with the mares running on a high-speed horse treadmill (Sato I; Equine Dynamics, Inc., Lexington, KY, USA) at a fixed 6% grade. The GXTs started at an initial speed of 4 m·s⁻¹ for 1 min. Speed was then increased to 6 m·s⁻¹, followed by incremental increases of 1 m·s⁻¹ every 60 s (omitting 5 m·s⁻¹) until the horses reached fatigue. Fatigue was defined as the point where the horse could not keep up with the treadmill despite humane encouragement.

**Blood sampling and real-time polymerase chain reaction**

Blood samples (30 ml) collected during all three trials were obtained via venepuncture into PAXgene™ Blood RNA Tubes (Qiagen, Inc., Santa Clarita, CA, USA) before exercise, immediately at the end of the test and at 30 min, 1, 2, 4 and 24 h post-GXT for the measurement of mRNA expression of IFN-γ, TNF-α and IL-6. Samples collected for the CON and EX-bute trials were also assayed for IL-1 and IL-10.

Total RNA was subsequently isolated from the tubes using spin columns according to the manufacturer's instructions. The RNA was quantified using a spectrophotometer (BioPhotometer; Eppendorf, Hamburg, Germany). In all cases, optical density (A₂₆₀/₂₈₀) ratios were greater than 1.9, and RNA yields were greater than 50 μg·ml⁻¹. One microgram of RNA was reverse transcribed into cDNA in an 80 μl reaction containing 20 units of AMV (avian myeloblastosis virus) RT, 0.5 μg of oligo dT primers, 40 units of RNAsin and 5 mM of MgCl₂ each cytokine and FAM (6-carboxy-fluorescein)-labelled probes for cytokines and β-actinase and primers based on the sequences for equine cytokines and β-glucuronidase (β-gus). Specific primers and FAM (6-carboxy-fluorescein)-labelled probes for each cytokine and β-gus, provided as Assay-by-Design kits (ABI), were added to 25 μl reactions in 96-well microplates containing the Taq polymerase (12.5 μl of Universal Master Mix; ABI) and 5 μl of cDNA. The following PCR conditions were employed: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected using β-gus as an internal control for each sample. Relative differences in cytokine mRNA expression resulting from exercise were determined by relative quantification. Relative quantification provides accurate comparison between the initial levels of target cDNA in a sample without requiring that the exact copy number be determined. The pre-exercise samples were selected as the calibrator, and the change in cytokine gene expression post-exercise relative to the calibrator was then determined for each sample.

**Statistical analysis**

Results are expressed as means ± standard error of the mean. For comparison by group and time, a two-way ANOVA for repeated measures was used with the *a priori* level of statistical significance set at *P* < 0.05. *Post hoc* comparisons were made using the Student–Newman–Keuls test (SigmaStat 2.0; SPSS, Inc., Chicago, IL, USA).

**Results**

Exercise caused increases (*P* < 0.05) in mRNA expression for TNF-α, IFN-γ and IL-6 (Fig. 1) that remained elevated through the 24 h collection point. There were no changes (*P* > 0.05) in mRNA expression for TNF-α, IFN-γ and IL-6 cytokines during the CON and EX-bute trials. IL-1 and IL-10 were measured only during the CON and EX-bute rounds of the experiment. There were no alterations (*P* < 0.05) across time, or due to exercise, in these two cytokines; therefore, those data are not shown.

**Discussion**

The cytokine responses to exercise in the horses of the present study appear to be similar to those documented for other species such as rats, mice and humans. Studies of humans have documented exercise-induced increases in plasma concentrations of TNF-α, IL-1β, IL-6, IL-1ra, IL-10 and IFN-γ during and after exercise, with the magnitude and duration of the response influencing the intensity, mode (eccentric vs. concentric) and duration of the exercise challenge. Other papers have documented that a variety of exercise challenges cause increases in urinary concentrations of TNF-α, IL-1β, IL-6, IL-2 receptors and IFN-γ.

Studies of horses have reported up-regulation of mRNA for TNF-α in horses during clinical situations where inflammation is present (e.g. arthritis, laminitis...
and obesity. This has also been shown to increase following experimentally induced inflammation where horses have been challenged with joint lesions or where they have been challenged with pathogens during vaccine development. Intense exercise has also been shown to cause an increase of TNF-α in humans and horses. Results have been mixed regarding the effects of exercise on IFN-γ. Most studies of humans and species other than horses have shown that excessively intense or long exercise causes an inhibition of IFN-γ production, with some papers suggesting that this leads to immunosuppression. However, it has also been reported that gene expression for IFN-γ, as well as the concentration of IFN-γ in plasma, is increased during the recovery from exertion. Baum et al. observed increases in IFN-γ mRNA expression 24 h after moderate exercise, but marked decreases in IFN-γ concentration levels 30 min after exhaustive exercise in humans, and thus suggested that the IFN-γ response is affected by the type of exercise performed. Interestingly, studies of horses have demonstrated that intense, short-term exercise causes an increase in IFN-γ mRNA expression in the immediate period following exercise.

The second finding of the present study was that phenylbutazone blocked the exercise-induced increase in cytokines seen in horses. This is a new and novel finding, since most studies of phenylbutazone that focused on exercise used in vitro experiments to examine the anti-inflammatory effects on more general systemic actions during exertion. Mechanistically, NSAIDs affect the inflammatory pathways in a variety of ways, with direct actions on cellular function and indirect actions on signalling between cells through endocrine, paracrine, autocrine and cytokine pathways. It is well documented that acute exercise induces changes in many components of the immune system, including increased expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. These cytokines are known to be involved in the regulation of the immune response and inflammation. Although the production of pro-inflammatory cytokines is believed to benefit the host defense system, the overproduction of these cytokines may lead to inflammation, subsequently causing muscle damage, weakness and increased risk of infection. There is also a systemic anti-inflammatory response tightly regulated by cytokines and characterized by changes in circulating leukocyte receptor and functional activity. It has been proposed that while a single bout of strenuous exercise produces a transient pro-inflammatory response, exercise training suppresses this inflammatory process, thereby contributing to the beneficial effects of habitual physical activity.

While anti-inflammatory medications and nutritional supplements have often been used to limit exercise-induced inflammation and soreness, the limited data available indicate that anti-inflammatory drugs largely have minimal influence on inflammatory responses to eccentric exercise and could lead to increased expression of some inflammatory markers. Phenylbutazone is routinely used in the horse for treatment of soft tissue inflammation and can be detected in plasma samples of racehorses as well as in their immediate environment. By inhibiting the cyclooxygenase enzyme system, which is responsible for synthesis of prostanoids such as PGE₂, phenylbutazone...
markedly reduces prostanoid-dependent swelling, oedema, erythema and hypersensitivity to pain in inflamed tissues. Like other NSAIDs, phenylbutazone inhibits TNF-induced NF-kB activation and IL-6 production. While the precise mechanism responsible for these effects remains unknown, this probably involves an non-cyclooxygenase activity of the drug.

The above speculation has focused on mechanisms behind the anti-inflammatory actions of phenylbutazone and how it can be a beneficial pharmacological intervention to counter the ergolitic effects of severe post-exercise inflammation. However, evoking a modulated up-regulation in inflammatory messengers may also be important for evoking an adaptive response to exercise. Many of the signalling pathways associated with the cytokine response to minor tissue repair are needed for increases in muscle strength and size. To that end, the increase in mRNA expression for the cytokines measured in the present study was rapid and of a magnitude consistent with what some have termed a 'beneficial inflammatory response' to acute exertion. Thus, one should caution against the prophylactic misuse of NSAIDs, as tone may actually inhibit those processes associated with tissue repair and muscle hypertrophy, as well as other cytokine-influenced systemic effects that are part of the normal exercise-induced response of the endocrine, cardiovascular and respiratory systems.

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