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Protein synthesis inhibition as a potential strategy for metabolic down-regulation

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Abstract

Objective—This pilot study tested the potential of puromycin (PUR) to inhibit protein synthesis and reduce oxygen utilization in a non-hibernating, whole animal preparation.

Methods—After anesthesia and instrumentation, male rats received a single dose of PUR or 0.9% saline (control), followed 60 min later with [³⁵S] methionine/cysteine radiolabeling. Thirty minutes after isotope injection, organ biopsies were taken for quantification of *de novo* protein synthesis. Arterial and central venous blood gases were obtained at baseline and 60 min after injection of PUR or 0.9% saline. Temperature, mean arterial pressure (MAP), and heart rate were recorded continuously.

Results—Animals receiving PUR demonstrated significant reductions in protein synthesis in all organ systems sampled ($p < 0.05$). The overall reduction averaged 67.8%. Central venous oxygen saturations (S_{cvO_2}) were higher in the PUR group than the controls at 60 min (90 ± 2 vs $80 \pm 4\%$, $p < 0.05$). The oxygen extraction ratio (O_2ER) decreased from 16.1 ± 1.7 to $6.8 \pm 1.2\%$ in the PUR group ($p < 0.05$) and increased from 12.5 ± 3.2 to $16.0 \pm 4.2\%$ in the controls ($p = 0.44$). There was no difference in temperature, MAP, heart rate or blood gas variables, other than S_{cvO_2} , at baseline or 60 min between groups.

Conclusions—These results demonstrate that PUR is capable of reducing whole body protein synthesis significantly within a relatively short duration of time. This appears to decrease whole body oxygen utilization as evidenced by an increase in S_{cvO_2} and a decrease in O_2ER . Protein synthesis inhibition may reduce metabolic demands and should be tested for its potential to improve outcomes where oxygen demands exceed oxygen delivery.

Keywords

Drug therapy; haemodynamics; oxygen; metabolism; shock

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1. Introduction

Shock is defined as a mismatch between cellular substrate delivery and utilization by the cell. Shock historically has been viewed as a problem with oxygen delivery rather than consumption and, therefore, most treatment strategies have focused on improving oxygen delivery. An alternative approach to the treatment of shock would be to reduce cellular metabolic needs, and thus whole body oxygen consumption. Production of this metabolically down-regulated state is envisioned to make the body more tolerant to acute pathologic states that are characterized by reductions in oxygen delivery to tissues.

Protein synthesis and proteolysis are high energy consuming processes and combined may account for nearly 45% of cellular ATP utilization [1,2]. Hibernating mammals and amphibians appear to use protein synthesis inhibition as a part of their strategy to reduce energy requirements and avoid ischemia in the face of reduced oxygen delivery [1,3–6]. Specifically, in their hibernating state, ground squirrels were found to have nearly undetectable levels of protein synthesis in the brain and other organs [6]. This down-regulated state is believed to be responsible for the ground squirrels' ability to tolerate low blood flow and reduced substrate and oxygen availability during hibernation. Additionally, it has been demonstrated that short-term inhibition of protein turnover does not appear to be deleterious [2,7–9]. In fact, protein synthesis inhibitors have been shown to reduce delayed neuronal death by preventing apoptosis [10,11], and providing neuronal protection against hypoxia [12] and oxygen-glucose deprivation-induced death [13].

Currently, the only clinical methods used to reduce metabolism are general anesthesia [14] and mechanically produced hypothermia [15]. Unfortunately, it can be difficult to employ these approaches in critical illness and injury. As a result of this difficulty, it becomes important to search for new methods that can be induced rapidly and easily in emergency situations such as trauma. Among the potential applications for this approach would be in combat casualty care, where the resources needed to improve oxygen delivery are scarce. A summary of critical research in the field of suspended animation as a therapy for the tolerance of complete systemic ischemia was presented at the International Resuscitation Research Conference in 1994 [16]. Part of this session included a call for a pharmacological or chemical induction of suspended animation in the field.

Puromycin hydrochloride is an aminonucleoside antibiotic produced by *Streptomyces alboniger* that inhibits protein synthesis at the level of translation [17]. Puromycin (PUR) was chosen not only for its potential effect on energy conservation by targeting translation, but also for the complete restoration of protein synthesis 30 minutes after removal of the drug [18]. Although many studies have evaluated protein synthesis inhibitors at a cellular or single organ level [2,12,13,19–21], to our knowledge there have been no studies that evaluate the effects of these agents on whole body protein synthesis and related oxygen utilization.

It was the purpose of this study to begin to understand to what extent protein synthesis could be inhibited in major organ systems by systemic intravenous application of PUR, and whether this was accompanied by evidence of reduced oxygen utilization.

2. Materials and Methods

This study was approved in advance by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. The animal facilities at this institution are approved by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC). The care and handling of animals were in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals" [22].

2.1. Animal preparation

Adult male Sprague-Dawley rats weighing between 300–400 grams were acclimatized for a minimum of five days prior to the experimental procedure. All animals were fasted but allowed free access to water during the night before the experiment. Animals were given a single intraperitoneal dose of ketamine (70 mg kg^{-1})/acepromazine (3 mg kg^{-1}) for induction of anesthesia, immediately followed by a subcutaneous dose of atropine sulfate (0.1 mg kg^{-1}) for control of secretions. Once unconscious, animals were placed on a heating pad in the dorsal recumbent position and a rectal thermometer was placed. Rectal temperature was recorded every 15 minutes throughout the experimental procedure. Attempts were made to maintain temperature at $37 \pm 0.5^\circ\text{C}$. The left femoral vein was cannulated in all animals with heparin-bonded single lumen PE-50 tubing using an open dissection technique. Anesthesia was maintained through the femoral venous catheter using a continuous intravenous infusion of alphaxalone-alphadolone starting at $0.05 \text{ mg kg}^{-1}\text{min}^{-1}$ and adjusted for a surgical plane of anesthesia. Animals were allowed to breathe room air and did not require artificial airway support. In a similar fashion, single lumen PE-50 catheters were placed in the left common carotid artery for continuous blood pressure monitoring and sampling intermittent blood gases, and the right internal jugular vein for administration of PUR or 0.9% saline (NS), radiolabeled amino acid, and sampling intermittent blood gases. Central placement of the internal jugular catheter in the superior vena cava near the right atrium was confirmed by the presence of negative deflections in the central venous pressure wave during rodent inspiration.

2.2. Measurements

Protein quantification of radiolabeled samples was performed on a Packard 1500 scintillation spectrometer (Downer's Grove, IL). Heart rate and mean arterial blood pressure (MAP) were measured continuously using BIOPAC AcqKnowledge®3.7.0 Data Acquisition System (BIOPAC Systems, Inc., Goleta, CA). Heart rate was determined from the arterial blood pressure tracings. Arterial and central venous blood gases were collected and analyzed for pH, pCO_2 , pO_2 , oxygen saturation and lactate (ABL 735, Radiometer, Copenhagen, Denmark) at baseline and 60 min. Oxygen extraction ratios (O_2ER) were determined using the formula $1 - (\text{S}_{\text{cvO}_2}/\text{S}_{\text{aO}_2}) \times 100$ [23,24], where S_{cvO_2} is central venous oxygen saturation and S_{aO_2} is arterial oxygen saturation. Rectal temperature was measured continuously and recorded every 15 min.

2.3. Experimental Procedure

Before the start of the experiment, baseline venous and arterial blood gases were collected and analyzed. Baseline temperature, heart rate and MAP were recorded. An intravenous dose of 250 mg kg^{-1} PUR in NS (Sigma, St. Louis, MO) or NS in equivalent volumes (control) was given. Sixty minutes after the administration of PUR or NS, repeat arterial and venous blood gases were drawn and the animals were injected intravenously with $750 \mu\text{Ci kg}^{-1}$ of [^{35}S] methionine/cysteine (ICN Biomedicals, Inc., Irvine, CA) to quantify *de novo* protein synthesis. Radioactive methionine was used to analyze *de novo* protein synthesis because it is well established that the initiation codon in protein synthesis is AUG which encodes for methionyl-tRNA [25,26]. Thirty minutes after isotope injection, animals were sacrificed painlessly by the intravenous injection of Euthasol (pentobarbital component approximately 200 mg kg^{-1}) followed immediately by double volume exchange transfusion, replacing arterial blood, withdrawn by rapid exsanguination via the carotid catheter, with NS chilled to 4°C . This technique was used to prevent any further uptake of isotope after the 30 min time period and to eliminate blood isotope activity.

2.4. Quantification of protein synthesis

At death, two biopsies were immediately taken from the liver, kidney, heart, lung, ileum, and skeletal muscle (right gastrocnemius). The first biopsy was weighed to approximately 50–60 mg, placed in 1 ml deionized-H₂O, snap frozen on acetone and dry ice and stored at –80°C. The second biopsy weighing approximately 100–200 mg was collected and placed on ice for determination of dry weight. Snap frozen samples were immersed in 37°C water bath for rapid thawing. Tissue was then homogenized using a micro tissue disrupter at 4°C. Trichloroacetic acid (TCA) was added to each homogenate to achieve a 5% concentration and the tubes were placed on ice for 5 min. The samples were centrifuged at 400x g for 10 min at 4°C to precipitate the protein. The supernatant was discarded and pellet was washed for 5 min with 2 ml of cold 5% TCA to remove unincorporated [³⁵S] methionine/cysteine until supernatant aliquots showed background radiation. After the final wash, the supernatant was decanted and pellet dissolved in 1 ml of 0.2M sodium hydroxide (NaOH). A 100 µl sample of the tissue/NaOH suspension was placed into a scintillation vial and 100 µl of 10% glacial acetic acid was added to adjust pH into the acidic range for accurate scintillation spectrometry. Five ml of scintillation fluor (Bio-Safe II, RPI, Mt. Prospect, IL) was added to each vial and samples were counted. Samples collected for dry weight were placed in a 90°C oven overnight. The dried specimens were weighed in a desiccated environment for wet/dry ratios. Data were corrected for weight and volume to give final numbers in disintegrations per minute/mg dry weight (dpm mg⁻¹).

2.5. Statistical analysis

Protein quantification of PUR and control groups, rectal temperature and oxygen extraction ratios were compared using paired 2-tailed *t*-test. Baseline and 60 min blood gas values, heart rate and MAP for PUR and control groups were compared using one-way analysis of variance (ANOVA); when a significant *p*-value was encountered, Newman-Keuls multiple comparison test was utilized for post-hoc comparisons. Statistical significance was defined as *p* < 0.05. Mean values were reported when noted with 95% confidence intervals. Otherwise, data are expressed as mean ± standard error of the mean (SEM). All statistical analysis was calculated using GraphPad Prism® Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

A total of 10 animals, five control and five PUR, were studied. There were no baseline differences between groups. Additionally, no significant differences existed in heart rate or MAP between groups throughout the experiment (Table 1).

3.1. Quantification of protein synthesis

Protein quantification of radiolabeled biopsy tissues from the control group averaged $4.7 \pm 0.6 \times 10^3$ dpm mg⁻¹, compared to tissues from animals given PUR ($1.5 \pm 0.3 \times 10^3$ dpm mg⁻¹, *p* < 0.05). The data from each tissue was used to determine the comparative decrease (by percentage) in protein synthesis at the end of the experiment. A significant decrease in the degree of protein synthesis was found in each organ system studied (Fig. 1). When compared to the control group, this represents an overall decrease in protein synthesis by 67.8%.

3.2. Blood gas analysis

Using the arterial and venous blood gases obtained at baseline and 60 min, the animals were screened for any changes in oxygenation and metabolic or respiratory abnormalities. There was no significant difference between baseline and 60 min within and between groups for pH, p_aCO₂, p_aO₂, S_aO₂, and lactate (Table 1). At 60 min, S_{cv}O₂ increased in the PUR group from 80 ± 1 to $90 \pm 2\%$ (*p* < 0.05), and decreased in the controls from 82 ± 2 to $80 \pm 4\%$ (*p*=0.64). O₂ER was used as a marker for whole-body tissue oxygen consumption. O₂ER decreased from

16.1 ± 1.7 to 6.8 ± 1.2% in the PUR group ($p < 0.05$) and increased from 12.5 ± 3.2 to 16.0 ± 4.2% in the controls ($p=0.44$, Fig. 2). This represents a 58% reduction in O₂ER in the PUR group versus a 28% increase in the control group ($p < 0.05$).

3.3. Temperature measurements

No significant differences in temperature were noted between groups prior to either PUR or vehicle injection. Despite our efforts to control rectal temperature with the use of a heating pad, the PUR injected group exhibited a statistically significant (0.5°C) drop in temperature, as opposed to the control group, which demonstrated an insignificant small (0.1°C) increase in temperature (Fig. 3).

4. Discussion

The main findings of this study are that protein synthesis inhibition by PUR significantly decreases whole body *de novo* protein synthesis and results in a concomitant increase in S_{cv}O₂ and decrease in O₂ER. These findings seem to support the hypothesis that there is a reduction in whole body oxygen consumption that accompanies protein synthesis inhibition. Additionally, the animals receiving PUR had similar hemodynamic and clinical variables to the control group indicating that this dose of PUR had limited immediate toxicity.

Oxygen debt is a major determinant in the outcome of shock states. Oxygen delivery (DO₂) can vary widely without changes in oxygen consumption (VO₂). VO₂ can be maintained in the face of reduced DO₂ by tissues increasing their O₂ER. This results in a decrease S_{cv}O₂ leaving the tissues. However, there comes a point where extraction in the face of decreased delivery cannot keep up with tissue oxygen demands. This point has been termed critical DO₂ (DO_{2crit}) [27] or dysoxia. It is at this point that tissues begin the conversion from largely aerobic metabolism to anaerobic metabolism. This is accompanied by significant increases in metabolic byproducts such as lactate. The degree and length of time at which VO₂ is dependent on DO₂ has been termed oxygen debt [28]. Accumulation of tissue oxygen debt has been demonstrated to correlate with the development of multiple organ system failure (MOSF) and death [29–31]. The potential to modulate the point of DO_{2crit} could be beneficial. By decreasing VO₂, the point of DO_{2crit} could be lowered thus potentially reducing oxygen debt prior to full resuscitation as well as reducing the resuscitation and post-resuscitation DO₂ needed to produce a state of VO₂ independent DO₂. If the lower oxygen consumption could make ATP available for other “lifesaving uses” within the cell, such as maintaining membrane ion gradients, this would conceivably result in a smaller oxygen deficit by delaying or reducing the magnitude of ischemic processes which contribute to cellular injury and death.

An additional theoretical benefit of this strategy is that the use of a protein synthesis inhibitor may reduce the degree of the reperfusion-induced inflammatory response by decreasing cytokine production. It has been shown that PUR can inhibit neutrophil function [32,33] and inhibit proinflammatory cytokines [34,35], all of which may require *de novo* protein synthesis. Of course, the additional actions of puromycin [36] do make determination of precise cause and effect mechanisms more complicated. However, we were unable to find an inhibitor of protein synthesis without other properties. We report here only one potential agent that inhibits protein synthesis. Other agents and combinations of agents may provide a more significant reduction in protein synthesis. Experiments in our laboratory combining another protein synthesis inhibitor, anisomycin, with puromycin showed an even greater inhibition of protein synthesis (80.2% inhibition, submitted for publication). Further studies are needed to examine other potential agents and to produce dose-response, timing, and safety profiles. We performed only one sampling time point and thus do not know if maximal protein synthesis inhibition occurs earlier or later in comparison to our sampling time, nor can we determine from this experiment when the maximal metabolic benefit may occur. An earlier study by Gorski et al.

[37] used a cumulative puromycin dose of 200–240 mg/kg over four hours (sequential hourly intraperitoneal injections of 15 mg puromycin into 250–300 g rats) compared to our single dose strategy employing 250 mg/kg intravenous administration. They demonstrated similar protein synthesis inhibition in the liver (85%), kidney (60%) and heart (64%). The protein synthesis inhibition was greater in muscle (90%), but uterine smooth muscle was used in that study as compared to skeletal muscle in the present study. There was no attempt to evaluate oxygen utilization following the multi-dose strategy in their study. The authors cited unpublished experiments indicating the dosing “schedule to be at a critical level” to inhibit protein synthesis. We have examined organ systems in rats one week after they received protein synthesis inhibitors in the doses used in this study. Animals did not exhibit any gross abnormal behaviors, weight loss or other signs of toxicity. Microscopic evaluation of tissues using hematoxylin and eosin staining has not produced any evidence of necrosis or other pathologic changes. However, chronic dosing with high doses of puromycin or lower doses of more potent aminoglycosides induces renal damage [38]. We did not examine the effects of PUR on brain protein synthesis in this study. However, subsequent studies using PUR in combination with anisomycin have demonstrated protein synthesis inhibition of up to 94% in the cerebrum. Lastly, we do not know the absolute amount of protein synthesis occurring in these animals. It is only the relative change from an anesthetized control that could be determined. It is possible that protein synthesis is already down-regulated to a degree in septic and traumatic shock [36].

The changes in $S_{cv}O_2$ and O_2ER were used to infer changes in VO_2 . The lack of changes in MAP and heart rate indicate that changes in DO_2 leading to alterations in $S_{cv}O_2$ and O_2ER are unlikely. Animals were kept on a heating blanket to maintain normothermia, thus limiting the ability to detect decreases in temperature caused by reductions in VO_2 . Nevertheless, rectal temperature actually dropped in the puromycin treated group, despite increasing the heating pad temperature in an effort to maintain normothermia. This supports the concept that the reduction in O_2ER was accompanied by a reduction in metabolism. Our group has begun experiments using a metabolic chamber to measure oxygen consumption and temperature in conscious animals. In addition, this treatment strategy should be studied in injury models where DO_2 and VO_2 are altered by the injury to better understand survival and timing limitations of the treatment.

After completion of these experiments, Blackstone et al. [39] reported on an alternate strategy to reduce metabolic rate. Their experiments involved the use of a cytochrome c oxidase inhibitor (hydrogen sulfide) combined with hypothermia to dramatically lower oxygen consumption. We did not attempt the combination of hypothermia with puromycin because the reduction in body temperature cannot be clinically extrapolated to humans with a 200-fold increase in mass and much larger thermal inertia (see *e*-letter by Christopher J. Gordon at <http://www.sciencemag.org>)

5. Conclusions

In this study, we provide evidence that the protein synthesis inhibitor, puromycin, is effective in decreasing whole body *de novo* protein. Simultaneously, puromycin increases $S_{cv}O_2$ and decreases the oxygen extraction ratio suggesting a decrease in oxygen consumption. One of the primary goals of resuscitation is to prevent the accumulation of oxygen debt, which has been directly related to mortality and development of multiple organ system failure. Metabolic down-regulation using protein synthesis inhibition may have the potential to provide tissue tolerance to reductions in oxygen delivery in shock.

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Fig 1.

A. Quantification of [³⁵S] methionine/cysteine-labeled protein expressed as disintegrations per minute per mg of dry weight (dpm mg⁻¹). B. Percentage of protein synthesis inhibition by organ biopsy. Values are mean ± SEM. **p*<0.05

Fig 2.
Oxygen extraction ratios (O₂ER) at baseline and 60 min in puromycin (PUR) vs control groups.
Values are mean $\pm$ SEM. * $p < 0.05$

Fig 3. Rectal temperature from baseline to 60 min post-injection in puromycin (PUR) vs control groups. Values are mean $\pm$ SEM. * $p < 0.05$

Table 1
Baseline and 60 min blood gas variables, mean arterial pressure (MAP), heart rate (HR) and temperature (Temp).

	pH	p _a CO ₂ (mmHg)	p _a O ₂ (mmHg)	S _a O ₂ (%)	S _v O ₂ (%)	Lactate (mmol/L)	MAP (mmHg)	HR (bpm)
Control								
Baseline	7.40 ± 0.01	33 ± 2	82 ± 6	94 ± 1	82 ± 2	0.7 ± 0.1	99 ± 8	390 ± 18
60 min	7.40 ± 0.01	30 ± 1	88 ± 6	95 ± 2	80 ± 4	0.5 ± 0.04	98 ± 6	415 ± 35
PUR								
Baseline	7.40 ± 0.02	30 ± 2	86 ± 8	95 ± 0.4	80 ± 1	0.7 ± 0.1	98 ± 6	382 ± 16
60 min	7.35 ± 0.01	28 ± 1	94 ± 4	97 ± 0.6	90 ± 2*	0.8 ± 0.2	90 ± 4	386 ± 4

Values are mean ± SEM.

*
p<0.05