Exercise-induced Stress Response as an Adaptive Tolerance Strategy

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Interaction between the quality of the environment and the health of the exposed population determines the survival response of living organisms. The phenomenon of induced tolerance by exposure to threshold levels of stressors to stimulate natural defense mechanisms has potential therapeutic value. The paucity of information on predictability of individual response and information on the operative fundamental mechanisms limit applicability of the adaptive tolerance strategy. A potential biomarker of the stress response includes members of the stress-inducible ubiquitin gene family. Transcript sizes detected with Northern blot analysis identify different classes of ubiquitin gene family members and the intensity of the radioactive signal allows abundance determinations. Using moderate exercise as the stressor, significant increase (p<0.028) in abundance of inducible polyubiquitin genes was found in human blood. Both the potential of exercise as a model system of a natural stress inducer and polyubiquitin as a biomarker of stress were established in these studies. — Environ Health Perspect 106(Suppl 1): 325–330 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-1/325-330smith_sonneborn/abstract.html

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Introduction

It has long been established that when organisms or cells are exposed to low levels of specific harmful chemical or physical agents, a beneficial physiologic effect is observed. Studies in Paramecium have provided evidence for increased longevity, cell division rate, and survival when exposed to low doses of ionizing radiation (1), ultraviolet (UV) radiation (2), electromagnetic radiation (3), and peroxide (4). Heat shock increases the lifespan in Drosophila (5) and C. elegans (6) and provides tolerance to subsequent thermal challenge. Heat-induced acquisition of therotolerance has been characterized in a broad range of organisms ranging from Escherichia coli to Homo sapiens, suggesting that this response is evolutionarily conserved and biologically important. Pretreatment with sublethal challenges of heat also has been implicated in acquisition of cross-tolerance to additional forms of insult including exposure to alcohol, anoxia, glutamate toxicity, reactive oxygen species, glucose deprivation, salinity, nutrient limitation, osmolarity, and endotoxins (7-11). From the perspective of the environment and health, the mechanisms involved in induced thermo- and chemotolerance may provide tolerance to the exposed population and transient protection against pollutants and/or environmental toxins.

It has been suggested that a mechanism contributing to the beneficial effects of otherwise harmful agents may operate through the stress response (SR) (11), a physiologic mechanism characterized by the induction of families of detoxification and repair molecules and reduction in general protein synthesis. The SR has been characterized using heat (the classical SR), radiation, heavy metals, and oxidizing agents (12,13). Treatment with specific stressors can confer cross-tolerance to a number of other chemical and physical agents.

The Stress Response and Cytotoxicity

When cells are exposed to heat or other toxins, abnormal protein folding directs the increased expression of inducible polyubiquitin and other heat shock proteins that can directly interact with the abnormal protein and assist in adenosine triphosphate (ATP)-dependent refolding or degradation. Exposure to sublethal challenges of stress may rejuvenate the cell by repairing damage before the challenge and may provide transient protection against further damage from subsequent sublethal or lethal challenges with a different otherwise harmful physical or chemical stressor (2,5,11).

In theory, any exposure to an agent that causes protein or DNA damage should induce the increased expression of stress-responsive proteins. Therefore, stress protein (SP) transcripts are also potential biomarkers of toxicity in microorganisms and possibly in the tissues of higher organisms. The research presented here investigates the potential of using exercise as the stressing agent for induction of preferential expression of stress-responsive ubiquitin transcripts in human blood.

Exercise-induced Stress Response

Stimulation of the SR by exercise may provide the following benefits: allow evaluation of the feasibility of exercise as an inducer of the SR and its potential therapeutic value; provide a model system for further investigation of the cytoprotective characteristics of a natural inducer of the SR; stimulate further investigation into mechanisms operating in exercise-induced health benefit; provide an adaptive strategy to induce tolerance to environmental pollutants; provide a potential biomarker for SR that could be adapted as a diagnostic tool for screening workers routinely exposed to toxic environments; and serve as a marker to monitor different exercise prescriptions for induction of the SR.

Some of the physiologic conditions experienced by cells of exercising animals known to induce SR include increased temperature, ischemia, reactive oxygen species, generation of abnormal proteins, uncoupling of oxidative phosphorylation, alterations in pH, alterations in calcium, and...
glucose deprivation (14). Most animal studies show increased production of the antioxidants superoxide dismutase and glutathione reductase (15) and heat shock protein, Hsp72 with exercise (16–20). Hsp70 family members are cytoprotective, especially in the heat shock response (12,13). Both antioxidant and SR defenses provide increased tolerance to environmental toxins.

Exercise effects will be monitored by examining the transcription profiles of the stress-inducible polyubiquitins, proteins in the pathway for cell survival, and potential blood biomarkers of the SR.

Cytoprotective Properties of the Ubiquitin System

Ubiquitin is a major stress-inducible transcript in mammalian cells (21) that is involved in pathway(s) for the repair of protein and DNA damage as well as the selective degradation of abnormal or misfolded proteins. The polyubiquitin gene is essential in yeast for resistance to high temperatures, starvation, and other stressors (22). Recently, exercise has been shown to induce SPS in a variety of tissues (16–20) and increase ubiquitin expression in muscle (23). The 76-amino acid ubiquitin protein is among the most highly conserved known and is identical among animals, varying with few exceptions by only three amino acids from yeast to humans (24). The classic function of ubiquitin in vivo has been its role in the ATP-dependent proteolytic pathway by conjugation to target proteins through a complex enzymatic system and tagging of these proteins for degradation (25). However, more recent work has suggested that ubiquitin conjugation may also play a regulatory role by acting on target proteins in a manner much like phosphorylation or methylation to alter the functional capacity of the target molecules (26). Ubiquitin has essential nonproteolytic functions in DNA repair, transcription, cell-cycle control, ribosome and peroxisome biogenesis, viral infection, apoptosis, development, and oncogenesis (21,27).

Two classes of ubiquitin genes have been identified in eukaryotes: ubiquitin fusion genes, which encode a single ubiquitin moiety fused to an unrelated ribosomal tail protein (28); and polyubiquitin genes, which consist of head-to-tail tandem repeats of complete or partial ubiquitin coding units (29,30). The ubiquitin fusion genes are expressed constitutively in the cell, whereas the polyubiquitin genes are induced in the SR or transcribed under specific physiologic conditions (21,27). In mammals the multigene ubiquitin family consists of genes and pseudogenes that are transcribed to produce three functional mRNA transcripts of approximately 0.65, 1.2, and 2.5 kb that are designated UbA, UbB, and UbC respectively (29,30). Although the precise function of these genes is not known, a change in transcription patterns may be a biomarker of stress.

The purpose of this investigation was to determine if abundance of polyubiquitin transcript increases in human blood following an acute bout of exercise using an anaerobic weight-lifting circuit.

Materials and Methods

Subjects

Subjects were recruited from participants in a local anaerobic weight-lifting circuit open to the general public. Volunteers’ initial activity levels were determined based on responses to a questionnaire of weekly activity. Most participants were classified as low- to moderate-intensity exercisers, exercising less than 30 min/day, 7 days/week, representing the 60% of the population that exercises but at levels below that recommended for health benefit (31). Multiple testing dates over a period of 2 years resulted in the following participant demographics: 7 men and 19 women with ages ranging from 32 to 75 (mean = 49 years of age). Participation in this study was strictly voluntary. All results presented reflect data collected from both novice and experienced volunteers (with a mean time interval in circuit training of 12 months).

This study was approved by the University of Wyoming Institutional Review Board for Human Subjects (Laramie, WY) and all procedures described below complied with established guidelines. All participants provided informed consent before participation.

Sample Collection

Participants in the acute study described in this paper volunteered to provide blood samples a) immediately before exercise to allow determination of baseline levels of transcription and to serve as untreated or unstressed controls; and b) immediately after exercise to examine changes in levels of transcription from control values. Blood samples were obtained by venipuncture after a 16-hr fast immediately before and after a single exercise bout in 10-ml sterile Vacutainer collection tubes (Gibco BRL, Gaithersburg, MD) containing 15% (w/v) K3-EDTA as preservative. Subjects were supervised by fully certified staff at exercise facilities at the University of Wyoming and a local exercise club, the Kourt House (Laramie, WY). Blood samples were processed as described below to quantify changes in the transcription of genes encoding members of the ubiquitin multigene family.

Exercise Regimen

The anaerobic weight-lifting circuit used for the study consists of 18 anaerobic (weight-lifting) stations interspersed with three aerobic stations (32) and provides significant beneficial long-term modulation of both cholesterol profiles and body composition in women and significant beneficial acute changes in cholesterol profile in men (33).

Circuit participation was individualized to maintain appropriate target heart rate and strength levels and conformed to the following parameters, which provide cardiovascular benefit for untrained individuals: a frequency of 3×/week at 20 min/session; a work-to-rest ratio of 2:1; and adjustment of loads to permit a maximum of 8 to 12 repetitions per station (34). Participants at higher conditioning levels routinely completed two circuits and used heavier weights than beginners. The exercise intensity was 70 to 80% of maximal heart rate estimated from the formula (220–Age)×0.70 or ×0.80. Heartrate was monitored throughout the exercise bout using a recording Vantage XL Heart Monitor (Polar, Port Washington, NY) to ensure compliance to the prescribed regimen.

Northern Blot Analysis

Tubes of blood were stored briefly on ice, separated by low-speed centrifugation, and processed immediately. The lymphocyte layer (buffy coat) was transferred to Tri-Reagent BD (Molecular Research Center, Cincinnati, OH), a phenol–guanidine thiocyanate monophosphate reagent permitting the rapid single-step isolation of total RNA from whole blood (35). Isolation and purification of lymphocytes before total RNA extraction was unnecessary for the following reasons: a) contemporary methodology for lymphocyte isolation is lengthy and could place enough stress on cells to produce a false SR; b) human erythrocytes are annucleated; and c) attempts at extracting total RNA from erythrocytes and serum with Tri-Reagent BD demonstrated that these components of whole blood did not contain measurable amounts of RNA in this laboratory.
Total cellular RNA was solubilized in diethyl pyrocarbonate-treated water, loaded onto a 1.25% formaldehyde-agarose gel, and separated by electrophoresis. Migration was visualized with a tracking dye containing bromophenol blue, xylene cyanol, and ethidium bromide. An RNA ladder (Gibco BRL) was also loaded to facilitate identification of individual polyubiquitin mRNA species. Gels were photographed under UV light and sample quality determined visually by the presence of two predominant bands of small (≈ 2 kb) and large (≈ 5 kb) ribosomal RNA. Separated total RNA was transferred to a nylon membrane (Amersham, Arlington, IL) by overnight capillary transfer in 20× sodium saline citrate (SSC) using standard methods (36). The blots were exposed for 4 min to a UV light source that delivers 600 μW/cm2 at a wavelength of 254 nm to cross-link RNA to the membrane. The blots were stored at room temperature before hybridization with radiolabeled DNA probes.

Plasmid pRB130 contains three complete ubiquitin repeats including the 3' untranslated region of the human UbB gene and was graciously supplied by R. Baker (37). The pUC19-based pRB130 vector was restricted with EcoRI (New England Biolabs, Beverly, MA). Radiolabeling the linearized plasmid amplified the signal strength of the pRB130 probe without excessive nonspecific binding or inhibiting hybridization to ubiquitin mRNA. The pUC19-based p19B vector was restricted with PstI (New England Biolabs) to free the 1-kb β-actin insert. Restricted fragments used for probing Northern blots were labeled with 32P dATP (New England Nuclear, Boston MA) using the Nick Translation System (Gibco BRL) according to manufacturer's specifications. Unincorporated nucleotides were separated from incorporated nucleotides by precipitation with ethanol and ammonium acetate. Signal strength of the precipitate was compared to that of the unincorporated nucleotides using a Geiger counter (Ludlum Measurements, Sweetwater, TX) to determine the degree of 32P dATP incorporation. Radiolabeled probes of high quality (probe to supernatant ratio > 2:1) were stored at −20°C before hybridization.

Blots were moistened in 20× SSC and incubated overnight in a formamide-based prehybridization/hybridization solution (Gibco BRL) according to the manufacturer's specifications. Radiolabeled probes were denatured by heating to 100°C for 20 min, added directly to the prehybridization solution, and blots incubated at 42°C for 24 to 48 hr. After overnight hybridization, blots were washed in the following solutions of increasing stringency: 5× SSC, 42°C, 2× 15 min; 1× SSC, 0.1% sodium dodecyl sulfate (SDS), 42°C, 1× 30 min; and 0.1× SSC, 0.1% SDS, 37°C, 1× 15 min (36).

Washed blots hybridized to radiolabeled probes for Northern analysis were exposed directly to scientific imaging film (Kodak, Rochester, NY) in X-Omatic cassettes with intensifying screens (Kodak) and stored at −70°C for a variable period of time determined by signal strength (18–240 hr). Autoradiographs were processed in developing solutions (Kodak) according to the manufacturer's specifications. Lengths of transcripts in kilobases visualized by autoradiography were determined using a logarithmic standard curve generated from migration distances of RNA fragments of known sizes (RNA ladder). Intensity of bands on autoradiographs corresponding to members of the ubiquitin multigene family was quantified by 2-D analysis using a laser densitometer. The ratios of mean values of UbB and UbC to UbA were calculated for quantitative and statistical analysis.

Statistical Analysis

A one-tailed paired Student's t-test was utilized to test for proportionate increased abundance in mean transcription ratios of polyubiquitin UbB and UbC to the non-changer UbA in control (pre) and exercise-stressed (post) samples. To evaluate correlates of change in ubiquitin transcription following an acute bout of exercise, simultaneous multiple regression analysis was undertaken. An alpha level of 0.05 was established as a criterion for statistical significance.

Results

Cellular ubiquitin transcription levels were determined by examining the ratio between the inducible polyubiquitin transcripts UbB and UbC and the noninducible gene, UbA, for pre- and postdensitometry values. UbA was used as nonchanging control for the following reasons. First, UbA is constitutively expressed gene containing promoter elements common to ribosomal housekeeping genes and is not induced by heat or other stressors (21,38). Second, statistical analysis showed a significant correlation between UbA and β-actin transcription (r = 0.75, p < 0.0001) with no significant difference between control and exercise-stressed UbA/β-actin ratios (p = 0.457). Third, UbA provided a good internal control that would allow standardization of polyubiquitin ratios between different blots and experiments (Figures 1 and 2, Table 1).

The proportionate increases in the polyubiquitins UbB alone, UbC alone, or the combination were determined from the Northern blot analysis of total RNA extracted from control and exercise-stressed blood lymphocytes. The exercise regimen was sufficient to induce a significant proportionate increase in the transcription of UbB and total inducible polyubiquitin UbB + UbC (p < 0.036 and p < 0.028). Increases in the relative abundance of UbC alone transcripts in stressed cells approached statistical significance (p < 0.057) (Table 1; Figure 2). The 4.8 transcript seen in Figure 1 was not routinely observed or analyzed.

Figure 1. Northern blot probed with 32P-labeled (A) human ubiquitin or (B) mouse β-actin immediately before and after an acute bout of exercise. Differences in UbA band intensity reflect different amounts of total RNA loaded in each lane when compared to (B) the control actin samples. Densitometry readings for this subject are found in Table 1, subject 7.
The increase in polyubiquitin transcripts (Table 1) differed between individuals. Two individuals exhibited UbB transcript and five subjects showed UbC increases greater than 1. Four individuals did not produce UbB in detectable concentrations in either pre- or postexercise samples. In contrast, UbC was produced at low levels in all but one subject.

Multiple regression analysis adjusted for age, time in circuit, and sex did not predict significant changes in transcript abundance in either UbB or UbC. Changes in polyubiquitin abundance are therefore attributable to other factors, most likely physiologic responses independent of age, time in circuit, or gender.

**Discussion**

Although the exercise response varied among individual participants, this regimen significantly increased the mean proportionate abundance of the polyubiquitin transcript UbB (and total polyubiquitin). The differences in abundance of polyubiquitin transcript between participants may be a reflection of initial conditioning level, intensity of exercise, hydration and/or nutritional state, mode of exercise, and individual genetic variations. UbC alone approached significant changes in abundance and may be expected to reach significance when greater numbers of determinations are available.

Previous human studies investigating the SR have focused either on elevated core temperature (19) or induced muscle damage (23), which could result in increased accumulation of denatured or damaged proteins. Other animal studies required exercise to exhaustion (17,18) before transcript increased and/or heat shock proteins (Hsp72, Hsp73, and Hsp90) were expressed in peripheral tissues (18). This study was unique in several ways. First, the induction of stress-responsive gene transcripts was found among participants exercising at moderate intensity levels. Second, the target population was neither elite athletes nor sedentary exercisers. Third, the transcription of stress-inducible polyubiquitin, a gene that plays a significant role not only in the SR but also in a wide variety of other essential cellular events, was significantly increased in human blood samples after exercise.

Previous studies with rats indicate that although repeated bouts of nonexhaustive exercise are sufficient to protect the heart from ischemic stress, one bout of exercise (60 min, treadmill running at 30 m/min) was not sufficient to elicit either cardioprotective effects or to induce heat shock proteins (16). However, findings from this study indicate that a single bout of exercise is sufficient to induce transcription of polyubiquitin in human subjects. These differences may be explained by the following: most previous studies have used primarily aerobic exercise regimens as the stressor whereas the aerobic weight-lifting circuit described above has a significant anaerobic component; the aerobic weight-lifting circuit is characterized by repeated periods of rest between stations; and this circuit was designed to work every major muscle group without specific emphasis on any one area. The protective effects of SPSs in skeletal muscle have not been investigated; however, it has been known for some time that a single bout of exercise can protect against future injury (39), which suggests a probable role for heat shock proteins. Immunologic cytochemical localization of ubiquitin conjugates have been found within rat red and white skeletal muscle tissue (40).

One unique observation is that several other studies have found that cells from aged animals demonstrate diminished SR (41,42), which is thought to compromise the ability of an adult organism or cells to survive episodes of stress (42-47). The present study did not find age to be a predictor of increased abundance of polyubiquitin.

Because there is little information about the function of the polyubiquitin transcripts UbB and UbC beyond an obvious

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### Table 1. Individual changes in relative polyubiquitin gene transcription following an acute bout of exercise.

| Subject | UbB pre | UbB post | UbB diff | UbB incr | UbC pre | UbC post | UbC diff | UbC incr |
|---------|---------|---------|---------|----------|---------|---------|---------|----------|
| 1       | 0.432   | 0.455   | 0.023   | 0.05     | 0.682   | 0.673   | -0.01   | -0.01    |
| 2       | 0       | 0       | 0       | 0        | 0       | 0.221   | 0.051   | -0.17    | -0.77    |
| 3       | 0.058   | 0.08    | 0       | 0.021    | 0.36    | 0.182   | 0.114   | -0.07    | -0.38    |
| 4       | 0.112   | 0.129   | 0.017   | 0.018    | 0.125   | 0.277   | 0.012   | 0.16     |
| 5       | 0.022   | 0.033   | 0.011   | 0.023    | 0.247   | 0.427   | 0.02    | -0.05    | -0.19    |
| 6       | 0.345   | 0.617   | 0.272   | 0.79     | 0.034   | 0.128   | 0.093   | 2.70     |
| 7       | 0.26    | 0.382   | 0.122   | 0.47     | 0.274   | 0.515   | 0.241   | 0.88     |
| 8       | 0.222   | 0.333   | 0.101   | 0.43     | 0.247   | 0.2    | -0.05   | -0.19    |
| 10      | 0.418   | 0.508   | 0.09    | 0.22     | 0.308   | 0.213   | -0.1    | -0.31    |
| 11      | 0.079   | 0.198   | 0.12    | 1.52     | 0.115   | 0.171   | 0.056   | 0.49     |
| 12      | 0.182   | 0.229   | 0.047   | 0.26     | 0.485   | 0.429   | -0.06   | -0.12    |
| 13      | 0.133   | 0.129   | -0.004  | -0.3     | 0.121   | 0.14    | 0.016   | 0.16     |
| 14      | 0.418   | 0.508   | 0.09    | 0.22     | 0.309   | 0.213   | -0.1    | -0.31    |
| 15      | 0.394   | 0.412   | 0.018   | 0.05     | 0.424   | 1.824   | 1.399   | 3.30     |
| 16      | 0.119   | 0.424   | 0.305   | 2.56     | 0.143   | 0.337   | 0.184   | 1.36     |
| 17      | 0.38    | 0.292   | -0.09   | -0.23    | 0.535   | 0.398   | -0.15   | -0.27    |
| 18      | 0.265   | 0.491   | 0.226   | 0.85     | 0.143   | 0.737   | 0.594   | 4.16     |
| 19      | 0.472   | 0.302   | -0.17   | -0.36    | 0.642   | 0.434   | -0.21   | -0.32    |
| 20      | 0.486   | 0.18    | -0.31   | -0.63    | 0.232   | 0.082   | -0.17   | -0.73    |
| 21      | 0.196   | 0.125   | -0.07   | -0.36    | 0.333   | 0.185   | -0.15   | -0.45    |
| 22      | 0.085   | 0.051   | -0.03   | -0.40    | 0.097   | 0.072   | -0.03   | -0.26    |
| 23      | 0.394   | 0.440   | 0.054   | 0.14     | 0.364   | 0.586   | 0.273   | 0.61     |
| 24      | 0.443   | 0.647   | 0.198   | 0.44     | 0.612   | 1.294   | 0.682   | 1.10     |
| 25      | 0.333   | 0.308   | -0.03   | -0.08    | 0.444   | 0.423   | -0.02   | -0.05    |
| 26      | 0.359   | 0.285   | -0.09   | -0.26    | 0.308   | 0.176   | -0.13   | -0.43    |

*Subjects exhibiting greater than 1 increase of UbB or UbC (or both). No significant differences in UbA and β-actin were found. Therefore, ratios of UbB and UbC concentrations were calculated relative to respective UbA pre- and postexercise values. The difference represents postexercise (post) versus preexercise (pre) values. The increase (incr) was calculated by difference (diff) divided by the preexercise ratio.*
role in replenishment of the free ubiquitin pool, interpretation cannot be made of the physiologic mechanism(s) involved in selective induction of these genes. However, evidence is accumulating that suggests a role for ubiquitin beyond ATP-dependent proteolysis.

Ubiquitination, like phosphorylation, is used in a wide variety of regulatory mechanisms and may be used to change the functional state of specific substrates. A regulatory role for ubiquitin is suggested by the reversible covalent modification by ubiquitination of substrates such as calmodulin, histones H2A and H2B, as well as several cell-membrane receptors (22,27,28); and by the fact that ubiquitin is required in a wide variety of cellular functions such as cell-cycle control, DNA repair, DNA replication, differentiation, peroxisome biogenesis, sporulation, and heavy metal resistance (21). Selective induction of polyubiquitin to replenish or increase the free ubiquitin pool under conditions of cellular stress could not only allow a cell to alter the function of critical repair molecules after conjugation with ubiquitin, but could also target selective molecules for degradation, repair accumulated protein and DNA damage, and provide transient elevated cellular defenses for the subject after exercise.

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