August 6, 1999

Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5660

Dear Sir or Madam:

I am writing to inform you that I have accepted an Assistant Professor position at East Carolina University School of Medicine effective June 1, 1999.

The project # N00014-96-1-0563, entitled "Expression and Function of Heat Shock Proteins in the Mammalian Gut during Experimentally Induced Hypoxia and Exogenous Stress" has been completed. Please see enclosed Final Technical report.

Sincerely yours,

[Signature]

Alexander K. Murashov, M.D., Ph.D.
Assistant Professor in Physiology
East Carolina University School of Medicine
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19990813 033
EXPRESSION AND FUNCTION OF HEAT SHOCK PROTEINS IN THE MAMMALIAN GUT DURING EXPERIMENTALLY INDUCED HYPOXIA AND EXOGENOUS STRESS

Murashov, Alexander K.

The research was focused on characterization of the role of heat shock proteins during exogenous stress using mouse as model. The results of experiments provided evidence that heat shock protein 25 (Hsp25) may be a specific factor protecting neuromuscular system from stress and stimulating subsequent regeneration. Hsp25 was specifically expressed in lower cholinergic motorneurons in normal conditions and was rapidly induced after heat shock and hypoxia...Hsp25 was colocalized with p38 and AKT kinases in the same cells. Specific p38 kinase inhibitor blocked expression of Hsp25 during regeneration. p38/AKT/Hsp25 cascade of signaling is critical for cell recovery.

Heat shock proteins, exogenous stress, hypoxia, regeneration, gut, neuromuscular system, Map kinases, AKT kinase, Hsp25
FINAL TECHNICAL REPORT

GRANT #: N00014-96-1-0563

PRINCIPAL INVESTIGATOR: Dr. Alexander K. Murashov (e-mail: am96@columbia.edu), Co-Investigator, Prof. Debra J. Wolgemuth (e-mail:djw3@columbia.edu)

INSTITUTION: Columbia University

GRANT TITLE: Expression and Function of Heat Shock Proteins in the Mammalian Gut During Experimentally Induced Hypoxia and Exogenous Stress

REPORTING PERIOD: 1 March 1996 - 1 June 1999

AWARD PERIOD: 1 March 1996 - 1 June 1999

OBJECTIVE: To characterize the role of the heat shock proteins (hsp) in the cellular response to hypoxic cell damage in the gut; to determine the function of the hsp in the cellular adaptation to hypoxic conditions and development of tolerance to subsequent hypoxic assaults.

APPROACH:
Hypoxia: Mice were subjected to hypoxia in a special chamber which provided a controlled atmosphere containing 6% oxygen and the balance made up of nitrogen for 2, 8, and 16 hours.
Hyperthermia: Mice were anesthetized with sodium pentobarbital (80 mg/kg body weight) and placed on a temperature-controlled heating pad (50°C). Body temperature was raised to 42°C and maintained for 15 minutes and the animals were allowed to recover for 2, 8 and 16 hours, followed by euthanasia with CO2.
Immunohistochemistry: After specified periods of time, control and stressed animals were euthanized with CO2 and perfused first with saline and then with 4% paraformaldehyde in PBS (pH 7.4). Tissues were postfixed in 4% paraformaldehyde, and then processed as paraffin or frozen sections.
Antibodies: Antibodies to hsp was purchased from StressGen Biotechnologies Corp. (Victoria, Canada). Immunostaining was performed using the Elite ABC Kit according to manufacturer's instruction (Vector Laboratories Inc., Burlingame, CA). Controls for specificity included: tissues processed without incubation in primary antibody, secondary antibody, or ABC complex solutions.

ACCOMPLISHMENTS: We have examined the spatio-temporal pattern of expression of members of the hsp cellular stress gene family at the protein level in the gut of adult mice subjected to experimental hypoxia and heat shock. Immunochemical detection in histological sections showed induction of the Hsp70 inducible family member in the mouse stomach after 2, 8 and 16 hours of hypoxia. The expression was observed in Chief cells, which secrete pepsinogen, and in Parietal cells, which secrete hydrochloric acid. Expression of Hsp32 (heat shock protein encoding Heme Oxygenase-1) was detected in the stomach at 8 hours after heat shock, in Parietal cells and in surface mucous cells of gastric pits. Expression of Hsp25 was induced in circumferential and longitudinal layers of the smooth muscles in all regions including the stomach, duodenum, small and large intestine. In the stomach, strong induction was also detected in squamous epithelium. In small intestine, the expression was restricted to Goblet cells, which secrete mucusogen and in lamina propria of villi. In lamina propria, the expression was localized to smooth muscle cells and lymphocytes. In large intestine, the expression of Hsp25 was detected in Goblet cells and Paneth cells, which secrete lysozyme. The results indicate that hsp is expressed in different cellular populations and in different patterns after hypoxia.
and heat shock. We also made the important observation that Hsp25 is specifically localized to primary motor neurons and fibers of the spinal cord and the brain stem, and is strongly induced after hypoxic injury. This indicates that Hsp25 can be a critical factor protecting function of mammalian gut at different levels including the smooth muscle and secreting cells, the fibers innervating them and motor neurons. Axotomy performed on sciatic nerve showed that Hsp25 is strongly induced in motor neurons on the 4th and subsequent days after injury and corresponds to the pattern of regeneration. The expression of Hsp25 co-localized with p38 kinase and Mapkapk-2 kinase and AKT. Injection of specific p38 kinase inhibitor blocked Hsp25 expression. Thus Hsp25 is implicated as a downstream factor of p38 Map kinase pathway which may play an important role in regeneration. Immunofluorescence on primary cell culture of sympathetic neurons showed co-localization of Hsp25 with F-actin in individual growth cones. This observation suggests that Hsp25 may be an actin binding protein. Hsp25 may promote axonal growth and regeneration through reorganization of actin filaments in nerve cells. AKT kinase a downstream member of PI-3 kinase pathway was also activated during regeneration. AKT plays an important role in protection from cell death. The pattern of expression of AKT recapitulated the expression pattern of Hsp25. Immunohistochemistry showed co-localization of AKT and Hsp25 to the same cell populations during regeneration. Furthermore, immunofluorescence on primary cell culture of sympathetic neurons showed co-localization of Hsp25 and AKT in individual growth cones.

SIGNIFICANCE: An understanding of the molecular mechanisms of cellular responses to hypoxia is a first step to identifying new methods for therapy of ischemic injury. Our studies showed that the cells of the mammalian gut with high secretory functions and functional load appeared to be particularly sensitive to oxygen depletion. Upregulation of Hsp25 at different levels of the gastro-intestinal system argue that this protein can be a critical factor required for protection and subsequent recuperation. These findings indicate that hsp25 can serve as molecular markers of hypoxic injury in different cellular populations of the gut, as well as predict the cell and tissues most likely to be affected by ischemia. Our findings further demonstrated that Hsp25 is an important factor for regeneration. Hsp25 promotes regeneration in injured cells by regulating actin cytoskeleton and interaction with p38 kinase and AKT kinase.

PUBLICATIONS AND ABSTRACTS:
REPORT OF INVENTIONS AND SUBCONTRACTS
(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THIS ADDRESS. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1. a. NAME OF CONTRACTOR/SUBCONTRACTOR:
   Columbia University
   N00014-96-1-0563
   b. ADDRESS (Include ZIP Code):
   630 West 168th Street
   New York, NY, 10032
   c. CONTRACT NUMBER:
   2. a. NAME OF GOVERNMENT/PRIME CONTRACTOR:
   b. ADDRESS (Include ZIP Code):
   c. CONTRACT NUMBER:

3. TYPE OF REPORT:
   a. INTERIM
   b. FINAL

4. REPORTING PERIOD (YYYYMMDD):
   a. FROM 19960301
   b. TO 19990601

SECTION I - SUBJECT INVENTIONS

6. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR "None" so state

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<th>DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER</th>
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<th>CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (x)</th>
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f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR

(1) a. NAME OF INVENTOR (Last, First, Middle Initial)
(2) a. NAME OF INVENTOR (Last, First, Middle Initial)

(b) NAME OF EMPLOYER

(c) ADDRESS OF EMPLOYER (Include ZIP Code)

SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACT AWARDED BY CONTRACTOR/SUBCONTRACTOR "None" so state

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SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR Not required if (X as appropriate)

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<tr>
<td>Murashov Alexander</td>
<td>Associate Research Scientist</td>
<td>[Signature]</td>
<td>08/05/1999</td>
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DD FORM 882, JAN 1999 (EG)