School of Medicine
Department of Physiology
East Carolina University
Brody Medical Sciences Building • Greenville, NC 27858-4354
252-816-2770 office • 252-816-3460 fax

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,

Alexander K. Murashov, M.D., Ph.D.
Assistant Professor in Physiology
East Carolina University School of Medicine
Brody Bldg, #6N-61
600 Moye Blvd,
Greenville, NC 27858
Phone: 252-816-3111
Fax: 252-816-3460
Email: amurashov@brody.med.ecu.edu

REPORT DOCUMENTATION PAGE

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1. REPORT DATE (DD-MM-YYYY)	2. REPORT DATE	3. DATES COVERED (From - To)
08.05.1999	Final	03/01/1996-06/01/1999

4. TITLE AND SUBTITLE
EXPRESSION AND FUNCTION OF HEAT SHOCK PROTEINS IN THE MAMMALIAN GUT DURING EXPERIMENTALLY INDUCED HYPOXIA AND EXOGENOUS STRESS

5a. CONTRACT NUMBER

5b. GRANT NUMBER
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6. AUTHOR(S)
Murashov,Alexander K.

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5f. WORK UNIT NUMBER

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Columbia University
630 West 168th Street,
New York, NY 10032

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The research was focused on characterization of the role of heat shock proteins during exogenous stressusing mouse as model. The results of experimets provided evidence that heat shock protein 25 (Hsp25) maybe 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.

15. SUBJECT TERMS
Heat shock proteins, exogenous stress, hypoxia, regeneration, gut, neuromascular system, Map kinases, AKT kinase, Hsp25

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Alexander Murashov
UU	UU	UU	UU	1	19b. TELEPONE NUMBER (Include area code) 252-816-3111

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 pentobarbitol (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 CO_2.
Immunohistochemistry: After specified periods of time, control and stressed animals were euthanized with CO_2 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 hsps were 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 mucinogen 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 hsps are 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 indicate 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 4^{th} 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 patter of Hsp25. Immonohistochemistry 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 hsps 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 :

1. Murashov, A.K. and Wolgemuth, D.J. (1996) Expression of heat shock proteins in the gut after hypoxia and exogenous stress. Abstract presented 9 September 1996, Cell Biology of Hypoxia, Naval Medical Research Institute, p. 13.
2. Murashov, A.K., Talebian, S. and Wolgemuth, D.J. Role of Stress Protein Hsp25 in Cellular Response of Motor Neurons to Stress and Injury. In: *Ischemic Stroke, IBC's 6^{th} Annual Conference, November 6-7, 1997, Washington, DC.*
3. Murashov, A.K., Ul Haq, I., Park, E. and Wolgemuth, D.J. Role of the Hsp25 and p38 kinase pathway in axonal regeneration of spinal motor neurons. In: *28^{th} Annual Meeting Society for Neuroscience, Los Angeles, CA, November 7-12, 1998, p.1845.*
4. Murashov, A.K., Talebian, S. and Wolgemuth, D.J. Role of stress protein hsp25 in the response of orofacial nuclei motor system to physiological stress. *Mol. Brain Research,* 1998, 63, 14-24.
5. Murashov, A.K., Talebian, S., Bauer, R., and Wolgemuth, D.J. Selective expression of different members of the heat shock family in the enteroendocrine cells of the gut after experimentally induced hypoxia and heat shock (in preparation).

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Aug 31, 2001

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

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Columbia University	N00014-96-1-0563			b a. INTERIM ☐ b. FINAL
b. ADDRESS (Include ZIP Code)	**d. AWARD DATE** (YYYYMMDD)	**b. ADDRESS** (Include ZIP Code)	**d. AWARD DATE** (YYYYMMDD)	**4. REPORTING PERIOD** (YYYYMMDD)
630 West 168th Street New York, NY, 10032	19960301			a. FROM 19960301 b. TO 19990601

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state) none

NAME(S) OF INVENTOR(S) (Last, First, Middle Initial) a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS (X) d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (X) e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
Murashov, Alexander K.	NONE	N/A		X		X		X

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER (Include ZIP Code)	(c) ADDRESS OF EMPLOYER (Include ZIP Code)		

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

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state) none

NAME OF SUBCONTRACTOR(S) a.	ADDRESS (Include ZIP Code) b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES (YYYYMMDD) f.	
			(1) CLAUSE NUMBER	(2) DATE (YYYYMM)		(1) AWARD	(2) ESTIMATED COMPLETION
None							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if: (X as appropriate)) SMALL BUSINESS or ☐ NONPROFIT ORGANIZATION X

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Murashov Alexander	Associate Research Scientist		08/05/1999

DD FORM 882, JAN 1999 (EG) PREVIOUS EDITION MAY BE USED. WHS/DIOR, Jan 99