REPORT DOCUMENTATION PAGE		Form Approved OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE 10/24/03	3. REPORT TYPE AND DATES COVERED 04/01/01 – ~~10/24/03~~ 31 Dec 03
4. TITLE AND SUBTITLE The Adaptive Response to Intestinal Oxidative Stress in Mammalian Hibernation		5. FUNDING NUMBERS DAAD190110455
6. AUTHOR(S) Hannah V. Carey, Ph.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin-Madison 2015 Linden Drive, Madison, WI 53706		8. PERFORMING ORGANIZATION REPORT NUMBER DUNNS number: 161202122
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211		10. SPONSORING / MONITORING AGENCY REPORT NUMBER ARO Proposal number 42419-LS .1

11. SUPPLEMENTARY NOTES
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12 a. DISTRIBUTION / AVAILABILITY STATEMENT	12 b. DISTRIBUTION CODE
Approved for public release; distribution unlimited.	

13. ABSTRACT . The goal of this project is demonstrate how mammalian hibernators utilize the physiologic consequences of metabolic depression, which include changes in mitochondrial function, low body temperatures (T_b) and reduced blood flow, to activate cellular signaling pathways that minimize oxidative damage to sensitive tissues during torpor-arousal cycles. Specific Aim 1 examines oxidative stress to the gut of ground squirrels during the seasonal cycle. (a) accumulation of oxidized lipids in the intestine (in progress); (b) seasonal changes in the intestinal glutathione redox system (completed); (c) seasonal changes in intestinal antioxidant enzymes (in progress). Specific Aim 2 examines consequences of intestinal oxidative stress during hibernation. a) seasonal changes in NF-κB activation in intestine and the identity of activated cells (completed); b) seasonal changes in the intestinal mucosal immune system (in progress); c) the effect of hibernation on enterocyte apoptosis and cell cycle regulators (in progress). Part c has already identified several pro-and anti-apototic pathways as well as cell cycle proteins that are activated in the gut during hibernation. We have also completed studies on protein ubiquitination and the response of the exocrine pancreas to hibernation. Other studies related to the main goal of the project are in progress, including development of an intestinal ischemia-reperfusion model to test the protective effects of the hibernation phenotype on this trauma-induced event. Our findings provide insight into the dynamic nature of the hibernating phenotype in terms of oxidative stress and development of antioxidant defenses during regulated hypothermia and hypometabolism, and contribute to our working model of "natural preconditioning" in mammalian hibernators.

14. SUBJECT TERMS Hibernation; oxidative stress; antioxidant defenses; redox balance; intestine; immune system	15. NUMBER OF PAGES 12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

THE ADAPTIVE RESPONSE TO INTESTINAL OXIDATIVE STRESS IN MAMMALIAN HIBERNATION
Department of Defense, Army Research Office. DAAD190110455

(1) List of papers:

van Breukelen, F. and H.V. Carey. 2002. Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels. *J. Comparative Physiology B* 172: 269-273.

Balslev-Clausen, A., J. M. McCarthy and H. V. Carey. 2003. Hibernation reduces pancreatic amylase levels in ground squirrels. *Comp. Biochem. Physiol. A* 134:573 – 578.

Carey, H.V., C.A. Rhoads and T.Y. Aw. 2003. Hibernation induces glutathione redox imbalance in ground squirrel intestine. *J. Comp. Physiol. B* 173:269-276.

Carey, H.V., M.T. Andrews and S.L. Martin. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews* 83: 1153-1181.

Papers presented at meetings:

Carey, H.V. and C.C. Fleck. 2001. Increased recruitment of intraepithelial lymphocytes into small intestinal mucosa during metabolic depression in ground squirrels. *Gastroenterology* 120:A319.

Fleck, C.C. and H.V. Carey. 2001. Activation of NF-κB in intestinal mucosa of hibernating ground squirrels involves activation of the IKK/IκB pathway. *FASEB J.* 15: A818.

Fleck, C.C and H.V. Carey. 2003. Expression of anti-apoptotic pathways in intestinal epithelial cells during hibernation. *FASEB J.* 17: A39.

Carey, H.V. Speaker and co-chair for symposium on "Cellular and Molecular Responses to Depressed Metabolism and Low Temperature". My presentation was entitled: "Stress-induced signaling pathways associated with depressed metabolism and low temperature"
Meeting: American Physiological Society Intersociety Conference on Comparative Physiology: Evolution, Adaptation and Application (San Diego, Aug 24-28, 2002)

(2) Scientific Personnel:

2001-2002:
Courtney Fleck, Ph.D. student. Stipend was not supported by grant but all research carried out by Ms. Fleck for her Ph.D. (supplies, equipment, etc.) is supported by this ARO award.
Melissa Lefton, Undergraduate student, worked as student hourly
Timothy Piazza, Research Specialist

2002-2003:
Courtney Fleck, Ph.D. student. Stipend was not supported by grant but all research carried out by Ms. Fleck for her Ph.D. (supplies, equipment, etc.) is supported by this ARO award.
Andreas Balslev-Clausen, visiting medical student from Univ of Copenhagen. Was supported by a Fulbright scholarship to work in our laboratory for 9 months in 2002 and participated in the ARO-funded project.

Julia McCarthy, undergraduate student, worked initially for academic credit, then for hourly wage on ARO-funded project
Jessica Reimer, Research Specialist; replaced by Katie Luterbach in July, 2003

(3) Report of inventions: N/A.

(4) Scientific Progress:
I. The following is a summary of the work proposed for this project and an update of the status of each section of the proposal as of October 23, 2003.

General Project Goals:

The long-term goal of this research program is to identify signaling pathways that link external stressors to cellular and molecular responses that provide tolerance to severe stress. The project uses ground squirrels as a natural model system to identify stress-induced defensive mechanisms in mammals. As a hibernating species, ground squirrels undergo each year profound changes in physiology that would be considered highly stressful for non-hibernating species such as humans. The specific goal of this research project is to test the hypothesis that the intestine is vulnerable to oxidative stress during the hibernation season, and as a consequence, activates defensive mechanisms that promote the maintenance of intestinal structure and function. A unifying theme of this research is that hibernators exploit the physiologic consequences of metabolic depression and low temperatures to induce cellular signaling pathways that minimize oxidative damage to sensitive tissues like the gut.

Aim 1: Effect of hibernation on intestinal lipid peroxidation, redox status and pro- and anti-oxidant enzymes.

a) Determination of oxidized lipids in intestinal mucosa: The tissue samples for these studies have been collected, processed and sent to our collaborator Craig Frank (Fordham University). The objective is to analyze intestinal mucosal samples for levels of conjugated dienes and malondialdehyde (the latter using the TBARS assay). We showed previously that conjugated dienes are increased in gut mucosa during the hibernation season, indicating increased lipid peroxidation. **Conjugated dienes are** intermediate compounds produced by peroxidation of polyunsaturated fatty acids (PUFAs). Levels of **malondialdehyde**, an end product of lipid peroxidation, estimate the amount of lipid peroxidation that *has already occurred* at the time a sample was collected. It is important that both of these analyses be done on the same tissues to fully assess lipid peroxidation because only the TBARS assay will reflect accumulation of lipid peroxidation products. The dual assays are nearly completed and data will be forthcoming by the end of January (at this point only the conjugated diene assays have been finished). The delay was due to technical difficulties with the TBARS assay that have occurred in the Frank laboratory. In addition to the lipid peroxide measurements, the mucosal samples are also being used to measure fatty acid composition in the tissues, which may reflect adaptive remodeling of plasma membrane bilayers during the hibernation season.

b): Characterize seasonal changes in intestinal glutathione (GSH) redox system: The experiments for this portion of the project have been completed and a manuscript based on the results is in press (*Journal of Comparative Physiology* B; please see accompanying manuscript). In brief, we found that the ratio of reduced GSH to its oxidized form (glutathione disulfide, GSSG), which is an index of oxidative stress, was 5-fold lower in hibernating compared with summer-active squirrels, a highly significant effect that is due primarily to elevated GSSG concentration in hibernators. During hibernation the total pool of GSH equivalents was lowest in squirrels undergoing arousal and highest in squirrels during interbout arousals, suggesting that one function of interbout arousals may be to restore

total glutathione pools in the gut. Hibernation decreased the activity of GSSG reductase by ~50%, which may account in part for the change in GSSG concentrations during hibernation. There were no significant differences in activities of glutathione peroxidase or glucose-6-phosphate dehydrogenase within the hibernation season or between hibernating and summer squirrels. However, a strong trend was observed for lowest values of both enzyme activities in squirrels shortly after entering torpor (ET group). This trend, if it is biologically significant, could indicate that the process of entering metabolic depression involves moderate release of ROS that are detoxified by GSH-related enzymes. Although seemingly paradoxical, we have previously observed similar trends for measures of oxidative stress to the gut, such as conjugated diene accumulation (5). Overall, this new study has demonstrated that hibernation in ground squirrels is associated with a shift in intestinal GSH redox balance to a more oxidized state. Although normally considered a pathologic change, the lack of overt damage to the intestine during the hibernation season suggests that this redox shift may be a component of the adaptive response to the hibernating state.

This study also showed that levels of the stress protein HSP70 in intestinal mucosa is highest in squirrels entering torpor and early in a torpor bout, and lowest in squirrels arousing from torpor and during interbout euthermia. We suggest that this pattern may reflect induction of the stress response as animals enter metabolic depression. Because HSP70 is well known in other systems to be a component of cellular and tissue preconditioning, expression of this inducible chaperone protein may be a mechanism that increases tolerance of intestinal tissue to subsequent stress that is imposed at other points in a torpor-arousal cycle, such as late in a torpor bout or during the arousal process.

There is growing evidence that stress proteins like HSP70 can be released to the extracellular environment following stressful events, and promote local inflammation through activation of the innate immune system (3). As we describe below, we have strong evidence that non-inflammatory activation of the mucosal immune system is an integral part of the response of the gut to torpor-arousal cycles. The possibly that extracellular HSP70 is induced during one or more stages in the hibernation season will be explored in our new studies.

c) Determine changes in other intestinal antioxidant enzymes: These studies are still continuing. Thus far we found that protein expression of copper-zinc superoxide dismutase (SOD) is very strong in the intestine, but the abundance of the enzyme (in terms of protein expression) does not appear to vary with season. We are currently measuring expression of manganese SOD, which may be more relevant to our project because it is particularly important in minizing damage to mitochondria during oxidative stress. Enzyme activity analyses and analysis of these and other intestinal enzymes that play a role in oxidative stress and antioxidant defense (xanthine dehydrogenase, xanthine oxidase, superoxide dismutase and catalase) are in progress.

Aim 2. Consequences of oxidative stress in the intestine during hibernation.

a) Determine seasonal changes in NF-κB activation and identify activated cells:

We have now confirmed that the strong NF-κB activation we observed previously in the gut of hibernating squirrels is not a gradual, seasonal change but instead must require the initiation of torpor-arousal cycles, because activation in fall animals just prior to the start of the hibernation season is similar to that observed in summer-active squirrels. Unfortunately, studies using immunohistochemical techniques to determine which cell types in the intestinal mucosa exhibit NF-κB activation during hibernation (i.e., enterocytes, immune cells, neurons, etc.) have continued to produce variable results due to high levels of non-specific binding of antibodies to intestinal tissues. However, when specific staining is observed it is clearly in the intestinal epithelial cells (enterocytes), with much less expression in mucosal immune cells. Thus, we conclude that NF-κB activation during hibernation is primarily an event that occurs in the enterocytes.

In addition to the immunohistochemical studies we have also examined several aspects of the NF-κB signal transduction pathway in hibernating and active squirrel intestine, to better understand what role this stress-activated transcription factor is playing in the hibernating phenotype. We have examined the expression of the NF-κB subunit p50 in cytosolic and nuclear extracts from intestinal mucosa because of the preponderance of p50-p50 homodimers in NF-κB proteins during hibernation (5). An example of a Western blot is shown below with quantitation of the signals from several animals per group, including three lanes representing cytosolic proteins from summer active squirrels (SA).

[For the following figure and others shown in this report, abbreviations are: **SA** or **ACT** =summer active ($T_b \approx 37$ °C); for the hibernation states, **EN**=entering into torpor ($T_b \approx 25$ °C); **ET**= early torpor, ≤ 24 h in deep torpor ($T_b \approx 4$ °C); **LT**=late torpor, ≥ 7 days in deep torpor ($T_b \approx 4$ °C), **AR**=arousing from torpor ($T_b \approx 25$ °C) and **IBA**=during interbout arousal ($T_b \approx 36$ °C).]

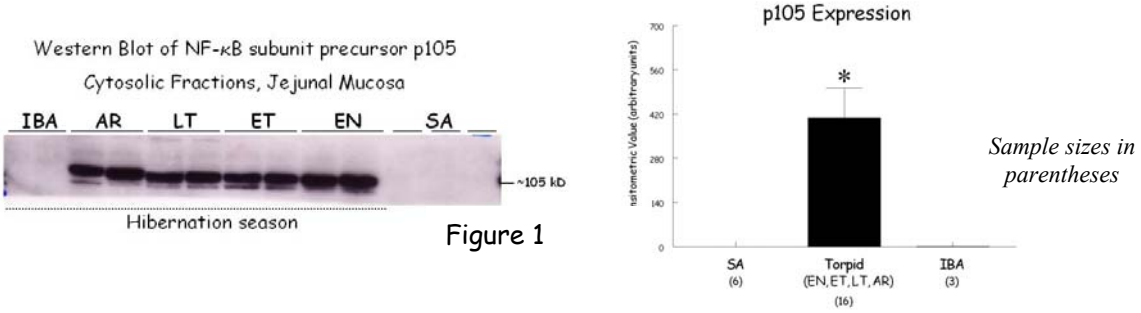


Figure 1

These experiments revealed that antibodies to the p50 subunit of NF-κB showed little cross-reactivity with proteins of ~50 kD molecular mass, but very strong staining for bands at ~100 kDa. Because these anti-p50 antibodies will cross-react with the precursor of p50, known as p105, this is likely the protein that is being recognized by the antibodies. As shown in the blot and the quantitation of Figure 1, this protein is very strongly expressed in hibernating squirrels, with much lower signals in the summer-active animals. It is also less prominent in interbout arousal squirrels that in squirrels at lower body temperatures.

Recent studies in other systems suggest that the p50 precursor, p105, binds to p50 homodimers in the cytoplasm and in so doing, retains them in the cytoplasm; thus, p105 appears to be the primary regulator of this NF-κB complex (rather than IκBα, which regulates p50-p65 heterodimers). It is possible that the strong expression of p105 all hibernation states except IBA may indicate that p50 homodimers are retained in the cytosol during torpid states but then released when p105 is degraded during arousals to euthermia. Degradation of p105 occurs secondary to stressful stimuli (e.g, TNFα) which, via activation of I-kappa-kinase induces phosphorylation and subsequent degradation of the protein via the ubiquitin-proteasome pathway (2). We suspect the process that leads to p105 degradation may be inhibited at low temperatures in torpid hibernators because of our studies on ubiquitin-mediated protein degradation during activity and hibernation (9). Thus, p105 likely accumulates in the cytosol during torpor states and then is degraded during interbout arousals. This possibility is currently being tested using immunoprecipitation experiments.

Two key downstream effects of activation of NF-κB are the development of the adaptive immune system and promoting or, more commonly, inhibiting apoptotic pathways. Thus, through induction of genes encoding cytokines, chemokines, enzymes and antimicrobial peptides, NF-κB helps to build the first line of defense against invading bacteria and fungi. For example, defects in NF-κB expression in rodents and humans is associated with microbial susceptibility. A wide variety of stimuli that activate pro-apopotic signaling pathways are antagonized endogenously by NF-κB-generated gene products. The following results from our project thus far implicate very strong associations with hibernation and changes in the mucosal immune system as well as apoptosis.

b) Determine seasonal changes in the intestinal mucosal immune system: These studies are designed to characterize the phenotypes of intraepithelial lymphocytes (IELs) in the intestinal mucosa of hibernating and active squirrels, in an effort to understand the function of the massive increase in IELs during the hibernation season. Because isolation of IELs for flow cytometry requires use of the entire intestine, we were not able to begin these experiments until the summer of 2002. That summer was used primarily to determine which antibodies would be most effective in identifying surface markers on IELs. Studies with intestinal tissue from active squirrels were completed this past summer (2003). In addition to the IEL population, we have now added characterization of two other mucosal immune populations: the lamina propria (LPL) and Peyers patch lymphocytes. The IEL population from six summer squirrels was comprised of ~70% αβ TCR lymphocytes and ~24% δγ cells. For 8 summer squirrels the LPL population was made up of ~71% αβ and ~16% δγ cells. These and other details of the lymphocyte populations will be determined in the hibernating group this winter and the two activity states will be compared. Together, our analysis of the three intestinal immune cell populations in hibernating and active squirrels will provide a complete profile of how this important barrier responds to the physiologic events associated with the seasonal hibernation cycle. To complement these flow cytometry studies on mucosal lymphocyte changes during hibernation, we have also begun analysis of seasonal changes in secretion of IgA molecules into the intestinal lumen. As IgA secretion is critical for defense against invading bacterial pathogens and to maintain the normal intestinal microflora, these studies will reveal how hibernation affects the body's ability to defend against invading pathogens, despite the long –term absence of food intake during the winter months.

b) Cytokine expression in intestinal mucosa and plasma samples. These experiments were designed to complement the IEL studies by examination of levels of specific cytokines that migh be affected by the changing mucosal immune system or be responsible for the changing mucosal immune system during hibernation. These studies began in winter of 2003 when sufficient numbers of mucosal samples were available. We are currently measuring expression of TNFα and IFNγ in hibernating and active ground squirrels.

Aim 2, Part c: **Determine effect of hibernation on apoptosis in the intestine**. This portion of the study began in early 2002. The TUNEL is being used as an index of apoptosis. Our quantitative results obtained thus far indicate a significant increase in TUNEL-positive enterocytes in the villi of hibernating squirrels (Figure 2), thus confirming the preliminary results that were presented in our original proposal.

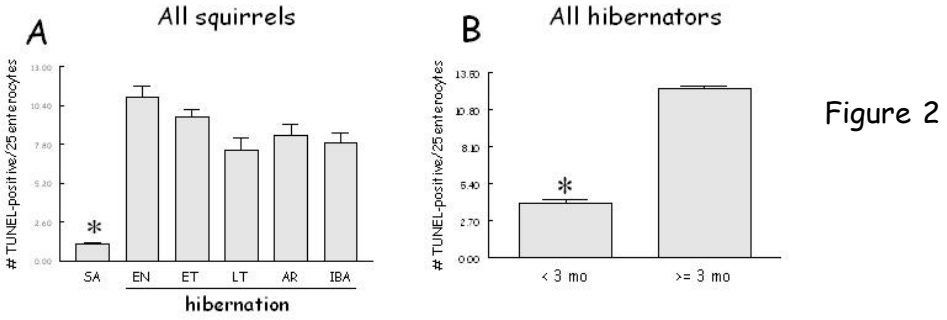


Figure 2

Quantitation of TUNEL-positive cells from 5 ground squirrel activity states is shown in Figure 2A (the T group represents ET and LT groups combined). Values in 17 summer active squirrels are significantly less than in any of the hibernation groups (n=55 total hibernators; $p<0.05$). Data obtained from our first year of animals suggested that for the hibernators, the numbers of TUNEL positive enterocytes appears to increase as the hibernation season progresses. Thus, hibernators from all groups were divided into early season (< 3 months of hibernation, completed ~8 torpor-arousal cycles, n=23)

and late season hibernators (> 3 months of hibernation, ~15 cycles, n=32) and a significant increase was observed in the late season animals. Thus, hibernation leads to accumulation of DNA damage (as indicated by TUNEL) as the season progresses. We feel these data are an important contribution to the hibernation literature, as there are no reports to date of apoptosis in any cell type.

Caution must be taken when using the TUNEL staining technique to confirm that the positive cells are indeed apoptotic and not undergoing some other kind of DNA modification. One way to test this is to examine differences in expression of proteins known to be involved in the apoptotic pathway, either as promoters or inhibitors of apoptosis. Our results thus far indicate that the pro-apoptotic proteins Bax and (activated) caspase 8 are strongly induced during hibernation compared with the summer-active state (Figure 3).

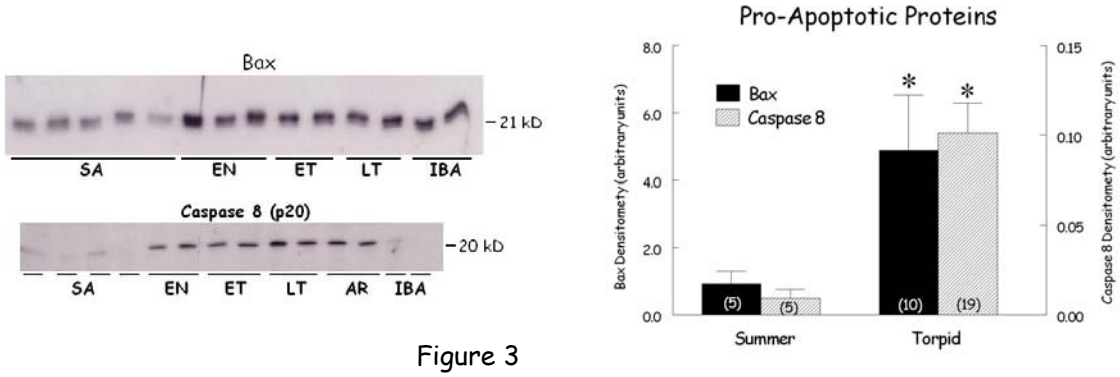


Figure 3

These results confirm that the TUNEL results do reflect activation of apoptotic pathways in enterocytes during hibernation. However, our working hypothesis is that the TUNEL staining results not full cell death, but damage to DNA (DNA strand breaks) that are in some way "rescued" during the hibernation season, thus preserving intestinal structure and function despite the stresses associated with chronic fasting and changes in tissue perfusion during torpor-arousal cycles.

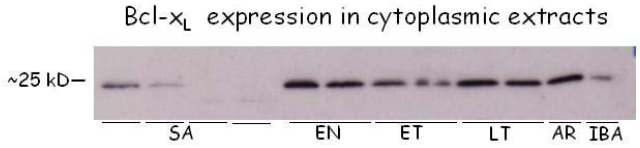
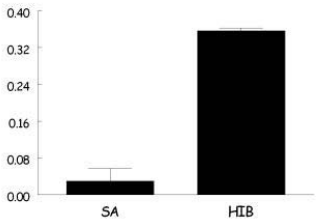


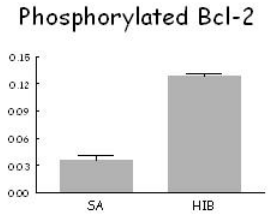
Figure 4



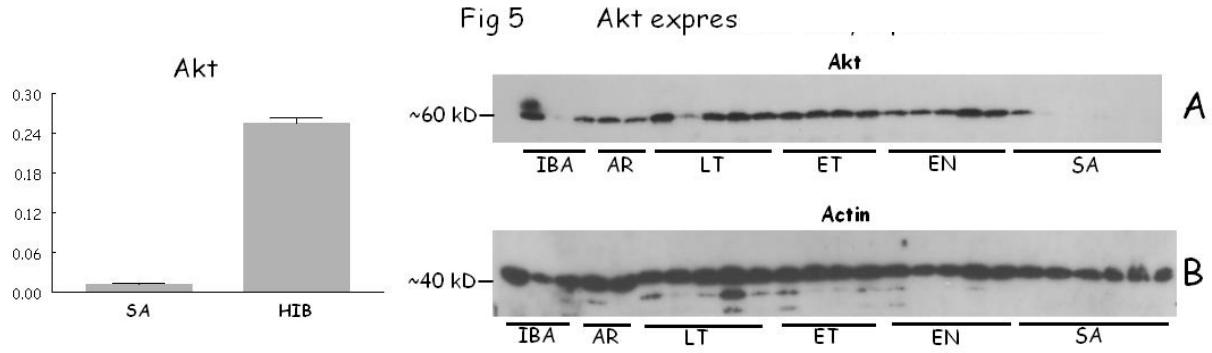
We hypothesized that to provide an adaptive response to counter hibernation–induced apoptosis in the gut, anti-apoptotic and other pro-survival proteins might be expressed. Our results thus far indicate that this appears to be the case (Figure 4). Bcl-x_L is an anti-apoptotic member of the Bcl-2 family that is transcriptionally regulated by NF-κB (7).

Bcl-x_L has been shown to block the release of cytochrome c from mitochondria in a variety of cell types, which otherwise occurs when cells are stimulated to undergo apoptosis. Studies in yeast suggest that Bcl-x_L expression may promote mitochondrial physiology by allowing for continued oxidative phosphorylation and maintenance of cellular ATP levels despite events such as decreased glycolysis (12). The greater expression of Bcl-x_L during the hibernation season strengthens our hypothesis that the intestine of hibernating squirrels responds to stress stimuli that might lead to cell death by increased expression of anti-apoptotic pathways.

Interestingly, although expression of another family member, Bcl-2, is not affected by hibernation, the phosphorylated form of Bcl-2 is increased. Phosphorylation is thought to enhance the anti-apoptotic effect of Bcl-2 in some cell types, thus it may play a defensive role in hibernators.



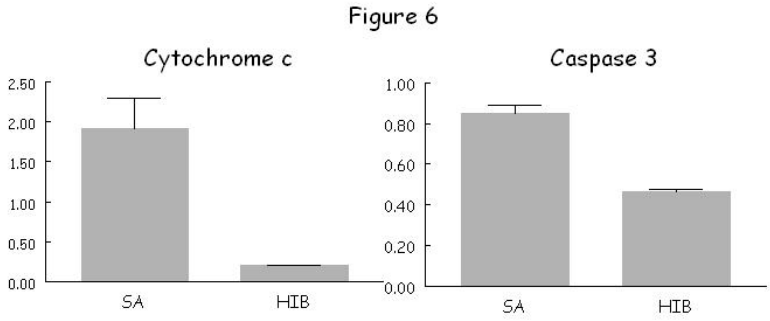
We have also identified Akt, a protein that is a downstream target of NF-κB, as another potential survival factor that is upregulated in enterocytes during hibernation. Akt, also known as protein kinase B (PKB), is an important regulator of several cellular processes, including proliferation, metabolism, and programmed cell death. As show in Figure 5A, Akt is expressed at higher levels in the hibernating groups compared with summer-active squirrels.



Also shown in Figure 5B is the same blot for Akt but probed simultaneously for actin; the results confirm that the absence of Akt signals in the summer lanes is not due to lesser amounts of total protein loaded in the gel, because actin signals are clearly evident in the summer animals.

There are a number of protein substrates that are affected by the kinase activity of Akt that have now been identified. For example, Akt's pro-survival effects on cells appears to be related at least in part to its ability to phosphorylate and thus inactivate the proapoptotic Bcl-2 family protein, BAD. Inactivation of BAD prevents its binding and inhibition of Bcl-x_L, thus increasing levels of free Bcl-x_L in mitochondria. Thus, the increased expression of Akt during hibernation provides a logical connection to our observations of increased Bcl-x_L expression at this time. We are very encouraged that our studies thus far have resulted in consistent and coordinated expression of pro-survival signaling mechanisms in the gut during hibernation.

Two mechanisms downstream of Bcl-x_L and Akt expression in terms of cell death and survival pathways are release of Cytochrome c and activation of caspase 3, both of which would promote apoptosis. We have found that both molecules are reduced during the hibernation season (Figure 6)



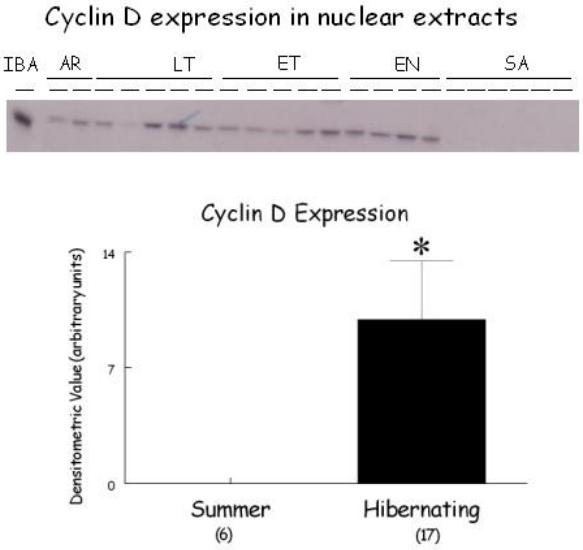
Given that pro-apoptotic gene products are increased during hibernation, and final effectors of apoptosis are reduced (caspase 3, cytochrome c) it is not clear whether the many TUNEL-positive cells we observe in enterocytes during hibernation actually represent cells that have fully undergone programmed cell death. In fact, our observation that expression of both pro- and anti-apoptotic genes can be detected in the same tissue samples has been reported previously for intestinal mucosa (1) and may represent not advanced stages of cell death but cell damage, which can lead to DNA fragmentation and TUNEL-positive staining of enterocyte nuclei (1). Bax and Bcl-2 (which is in the same gene family as Bcl-x_L) were found to be co-localized in small intestinal mucosa of mice. Based on their data and that of others, the authors suggested that sublethally damaged cells, as indicated by Bax expression, may upregulate Bcl-2 as part of a protective mechanism. Interestingly, in yeast Bax expression induces mitochondrial lipid peroxidation, and this effect leads to cell death but is prevented by co-expression of Bcl-x_L (8). Taken together with our own studies on lipid peroxidation during hibernation (5), these observations suggest that the Bcl-x_L may play a functionally relevant role in countering Bax-induced modifications to mitochondria and cellular apoptosis in hibernator.

Increased production of ROS can both induce antioxidant defenses and damage macromolecular structures in cells, including mitochondrial DNA. Because our previous studies showed that intestinal tissues of hibernators are fully functional when tissues are warmed to euthermic temperatures (4, 6), the most likely conclusion from our current data at this time is that sublethal damage occurs to enterocytes during the hibernation season, but protective mechanisms are activated to maintain intestinal health. Such mechanisms include expression of anti-apoptotic, pro-survival proteins such as Bcl-x_L and Akt. Our current data are not inconsistent with the idea that one or more events that are associated with hibernation lead to DNA damage, including nuclear and/or mitochondrial DNA, and the activation of the apoptotic program is a manifestation of this effect. Our working hypothesis is that the interbout arousals during the hibernation season represent euthermic intervals when defense mechanisms are operational, and in so doing rescue cells from the execution of cell death pathways, lead to macromolecular repair, and enhance cellular and tissue survival. To test this idea, in new studies just initiated we are examining DNA damage (DNA laddering) and expression of DNA repair mechanisms during activity and hibernation in ground squirrel intestine. Differences in oxidative DNA modifications between summer-active and hibernating squirrels of different activity states are being examined in immunocytochemical experiments using antibodies to the DNA damage marker 8-OHdG, which is one of the most abundant lesions in DNA generated by oxidative stress. We are using the DNA laddering technique to obtain more precise information on effects of hibernation on DNA stability than is possible with the TUNEL technique. We are also using antibodies raised against proteins involved in DNA repair in Western blotting and immunochemical studies, including p53 and PARP-1.

c) Examination of cell cycle regulators in hibernating and active squirrels. Because the promoter region of the cyclin D gene includes binding sites for NF-κB, we hypothesized in our original proposal that one consequence of NF-κB activation during the hibernation season may be the modulation of epithelial cell proliferation during entrance into torpor and the periodic arousals to euthermia. The absence of food intake during the hibernation season, as well as the temperature effect (Q_{10}) on suppressing protein transcription and translation (10, 11) are strong inhibitors of intestinal mucosal growth. This has the potential to severely compromise nutritional capacity in the spring after terminal arousal. Thus, interbout arousal periods provide a euthermic period when cell proliferation can resume and help counter the loss of absorptive tissue. Factors that would promote cell cycle entry and proliferation during IBAs are therefore likely to be favored, and strong stimulation of cell cycle regulatory proteins via NF-κB activation would promote this effect. Through inhibiting apoptosis and promoting cell proliferation, NF-κB can act as a regulator of multiple effects on the intestine that help promote integrity, prevent bacterial translocation, and maintain mucosal mass at a basal level so that food digestion and absorption can occur after terminal arousal. Our results thus far indicate that cyclin

D1 is indeed differentially expressed during the hibernation season. Cyclin D1 protein expression is strong during all phases of hibernation and particularly so during interbout arousals (Figure 7).

Figure 7



II. Other relevant studies:

A. Ubiquitination of intestinal and liver proteins in hibernating and active squirrels. The manuscript based on this work has now been published (see van Breukelen, F. and H.V. Carey, 2003, in list of published papers).

B. Effects of hibernation on pancreatic amylase levels in ground squirrels. (see Balslev-Clausen, et al., 2003, in list of published papers). Exocrine pancreatic activity is critical to GI function to provide enzymes for luminal hydrolysis of dietary components. Although the physiology of the endocrine pancreas in hibernators has received significant research attention, our knowledge of how hibernation affects pancreatic exocrine function (which represents 95% of total pancreatic mass) was previously unknown. Using pancreatic amylase as a representative enzyme, we found that enzyme activity and protein levels are reduced 40%-50% during hibernation compared with the summer, fed state. Although this is a significant reduction, given the length of time squirrels are fasted during the hibernation season this represents a remarkable conservation of amylase levels. In non-hibernators such as rats, just 3 days of fasting leads to similar reductions in amylase levels. Thus, this study is an important complement to our understanding of the response of the digestive system to the long-term fast of hibernation.

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