Modulation of apoptotic pathways in intestinal mucosa during hibernation

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Submitted 14 February 2005; accepted in final form 7 April 2005

Fleck, Courtney C., and Hannah V. Carey. Modulation of apoptotic pathways in intestinal mucosa during hibernation. Am J Physiol Regul Integr Comp Physiol 289: R586–R595, 2005—Mammalian hibernation is associated with several events that can affect programmed cell death (apoptosis) in nonhibernators, including marked changes in blood flow, extended fasting, and oxidative stress. However, the effect of hibernation on apoptosis is poorly understood. Here, we investigated apoptosis and expression of proteins involved in apoptotic pathways in intestinal mucosa of summer and hibernating ground squirrels. We used terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to identify possible apoptotic enterocytes in small intestine of summer squirrels and hibernating squirrels throughout the winter. Nuclear TUNEL staining increased as hibernation progressed, but the staining pattern was diffuse and not accompanied by chromatin condensation or apoptotic bodies. Electrophoresis of mucosal DNA revealed no ladders typical of apoptosis. Nuclear levels of proapoptotic p53 protein were fourfold less in hibernators compared with summer squirrels. A 12-fold increase in anti-apoptotic Bcl-xL compared with a 2-fold increase in proapoptotic Bax suggested a balance in favor of antiapoptotic signaling in hibernators. There was no change in Bcl-2 protein expression but phospho-Bcl-2 increased in mucosa of hibernators. Hibernation had minimal effects on expression of active caspase-8 or -9, whereas caspase-3-specific activity was lower in hibernators during an interbout arousal compared with summer squirrels. Expression of the prosurvival protein Akt increased 20-fold during hibernation, but phospho-Akt was not altered. These data provide evidence for enhanced expression of antiapoptotic proteins during hibernation that may promote enterocyte survival in a pro-oxidative, proapoptotic environment.

Bcl-xL; phospho-Bcl-2; p53; Akt; caspases

APOTOSIS, OR PROGRAMMED CELL death, is a process whereby damaged or defective cells are eliminated from tissues with little detriment to surrounding cells. Two main cellular signaling pathways have been identified in the apoptotic program. The intrinsic pathway is induced primarily by endogenous stressors, such as oxidative stress, that lead to increased permeability of the outer mitochondrial membrane and release of cytochrome c from the intermembrane space (34, 42). The extrinsic pathway is activated by the binding of specific ligands, such as TNF-α, to receptors on the cell surface (42). Both the intrinsic and extrinsic pathways lead to the activation of a highly conserved family of cysteine proteases called caspases. Caspases initiate a series of events that culminates in DNA cleavage and condensation, cytoskeletal disassembly, membrane blebbing, and cell death (42). Controlled levels of apoptosis are essential for some physiological functions, such as development; however, too much or too little apoptosis can lead to a variety of pathological conditions, such as degenerative diseases and cancer (15). Apoptosis has been extensively studied in cultured cells and in intact tissues from a variety of animal models of disease and protection. However, few studies have examined apoptosis in species that routinely undergo physiological changes typically associated with proapoptotic stimuli.

Mammalian hibernation is a striking adaptation used by some animals during periods of relative food scarcity and harsh environmental conditions (33). During the hibernation season, animals spend prolonged periods in torpor, during which body temperature (Tb) is low and metabolic rate is only a few percentage of normal. Torpor bouts are interrupted by periodic arousals when Tb and metabolism return to summer levels. Several aspects of the hibernation phenotype would be considered stressful for nonhibernating species. For example, during entrance into torpor blood flow is shunted away from splanchnic organs and is returned during arousal, which increases the potential for transient ischemia-reperfusion events (2). For seasonal hibernators like the 13-lined ground squirrel (Spermophilus tridecemlineatus), winter is accompanied by an extended fast and reliance on fat stores accumulated during the late summer and fall (4). Hibernation and the associated torpor-arousal cycles therefore have the potential to induce physiological stress to sensitive organs, including the intestine (6), which is the focus of the present study.

In mammals, stem cells of the intestinal crypts normally experience a low level of apoptosis to eliminate mutated or defective cells and control overall cell numbers (24). In contrast, apoptosis of differentiated enterocytes on the villus is rare under normal conditions but increases in response to a number of pathological and stressful conditions, including lack of luminal nutrition (14, 20, 23), ischemia-reperfusion (37), and oxidative stress (34). Given the similarities between these conditions and the physiological changes associated with hibernation, we hypothesized that enterocyte apoptosis would increase in hibernating compared with summer squirrels. Thus we studied apoptosis in intestinal mucosa of summer ground squirrels and in five activity states during the hibernation season. We analyzed the level of DNA fragmentation and the expression of proteins involved in the intrinsic and extrinsic apoptotic pathways. We determined the expression of several Bcl-2 family members that either inhibit or promote cytochrome c release from the mitochondria (42). We assessed the expression of active caspases, including caspase-9, caspase-8, and caspase-3. We also examined nuclear levels of p53 (42), which can induce apoptosis in response to extensive DNA damage (38, 43), and total and phosphorylated forms of Akt.

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which can inhibit apoptosis through several mechanisms (45, 51).

METHODS

Animals. Adult 13-lined ground squirrels of both sexes were trapped in the vicinity of Madison, Wisconsin, in summer (July-September). Squirrels were housed individually with free access to water and food (Purina rodent chow 5001, supplemented with sunflower seeds) at 22°C with a 12:12-h light-dark cycle. Squirrels were held in these conditions for at least 1 mo before use in experiments. In September, squirrels were transferred to a room maintained at 4°C. The room was dark except for brief periods (<20 min) of low lighting once per day to check activity state. Water and food were removed after squirrels began regular bouts of torpor. The University of Wisconsin Institutional Animal Care and Use Committee approved all animal procedures.

Telemetry. In mid-August, squirrels were implanted with temperature-sensitive radio telemeters (VitalView S3000, Minimitter, Bend, OR). Squirrels were anesthetized with 5% isoflurane, and a small midline incision was made in the skin and the abdominal muscle. The previously sterilized telemeter was placed inside the abdominal cavity, and muscle and skin incisions were closed. Telemetered animals were transferred to the cold room at least 1 mo after surgery, at which time Tb was monitored every 2 min using the Minimitter VitalView system.

Tissue collection. Squirrels were killed by decapitation. Summer squirrels were killed in early August when Tb was ~37°C. Activity states during hibernation (October-March; Fig. 1) were as follows: (1) entrance, entering torpor (Tb = 20–25°C); (2) early torpor, ~24 h in torpor (Tb ~5–7°C); (3) late torpor, ~7 days in torpor (Tb ~5–7°C); (4) arousal, arousing from torpor (Tb = 20–25°C); and (5) interbout arousal, active between torpor bouts (>3 h at Tb ~37°C). Hibernating squirrels of each activity state were killed early (~3 mo) or late (≥3 mo) in the hibernation season. Jejunum was removed and placed in ice-cold 0.01 M PBS. A 1-cm segment was fixed in 10% buffered formalin for 1–2 h and processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Mucosa was scraped from the remaining tissue. A portion (100–200 mg) was homogenized in a buffer containing 10 mM HEPES, 0.1% Triton X-100, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, and protease inhibitor cocktail. The homogenate was centrifuged at 5,000 g for 10 min, and the supernatant containing cytosolic proteins was collected. The pellet was resuspended in a buffer containing 20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, and 25% glycerol. After 30-min incubation on ice, samples were centrifuged at 14,000 g for 30 min, and the supernatant containing nuclear proteins was collected. Purity of nuclear proteins was confirmed by absence of immunoreactive sucrase-isomaltase, a brush-border enzyme normally found only in plasma membrane and cytosolic fractions. The remaining mucosa was frozen in liquid nitrogen.

TUNEL assay. The in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN) was used for TUNEL detection of DNA fragmentation. Deparaffinized sections were incubated with 3% H₂O₂ to block endogenous peroxidases, washed, and treated with proteinase K (20 µg/ml, 37°C, 15 min). Sections were incubated with fluorescein-conjugated nucleotides and terminal deoxynucleotidyl transferase (1 h, 37°C). After application of anti-fluorescein antibody, sections were treated with diamobenzadine. Four fields containing intact villi were chosen per section, and 25 adjacent enterocytes in the midvillus region were selected. A different individual then recorded the number of TUNEL-positive nuclei within the 25 enterocytes. The midvillus region was defined as those cells that were at least 10 enterocytes from the mouth of the crypt and 10 enterocytes from the villus tip. The specificity of TUNEL for DNA (not RNA) was confirmed by RNase digestion (20 µg/ml for 15 min at 37°C) following proteinase K treatment; this did not affect the degree of positive staining in any group.

DNA laddering. We isolated DNA from 100–200 mg of mucosa using the apoptotic DNA ladder kit (Roche Diagnostics). Mucosa was homogenized in a binding/lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4). Samples were then centrifuged (8,000 rpm, 1 min) to pellet mucus and debris. DNA was extracted from the supernatants by use of glass fiber fleece filters. The filters were then washed three times (20 mM NaCl and 2 mM Tris-HCl in ethanol, pH 7.5). DNA was eluted from the filters with a 10 mM Tris buffer (pH 8.5, 70°C). DNA was loaded onto a 2% agarose E-gel, run on the E-gel Power Base (Invitrogen, Carlsbad, CA), and then visualized on a UV transilluminator. The specificity of TUNEL for DNA (not RNA) was confirmed by RNase digestion (20 µg/ml for 15 min at 37°C) following proteinase K treatment; this did not affect the degree of positive staining in any group.

Immunoblotting. SDS-PAGE was performed on cytosolic or nuclear fractions (40 µg) from mucosa, and the expression of apoptotic proteins was determined by Western blotting. Antibodies included anti-caspase-8 (sc-7890), anti-Bax (sc-7480), anti-Akt1 (sc-1618), and anti-phospho-Akt1 (sc-7985-R) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bcl-2 (no. 554807) from BD Pharmingen (San Diego, CA); and anti-Bcl-XL (no. 2762), anti-phospho-Bcl-2 (no. 2871), anti-caspase-9 (no. 9506), and anti-p53 (no. 2524) from Cell Signaling Technology (Beverly, MA). The anti-phospho-Bcl-2 antibody was specific to human protein; all other antibodies were cross-reactive with mouse, rat, and human. Antibodies were used only if they produced a single strong band at the appropriate molecular mass; this was the case for all antibodies except anti-caspase-9, which also revealed a weaker cleavage product that was not quantified. We analyzed protein bands using ImageQuant software (Amersham). Cytosolic proteins were normalized to Hsc70 (no. SPA-816, StressGen, Victoria, BC), a constitutively expressed heat shock protein, and nuclear proteins were normalized to actin (both were previously determined to be representative of protein loading based on Ponceau
S staining), Akt, phosphorylated form of Akt (pAkt), and p53 blots were stripped and reblotted to determine Hsc70 or actin levels. All other blots were cut at ~50 kDa and probed for both Hsc70 and the protein of interest.

Statistics. Group comparisons were analyzed by one- or two-way ANOVA where appropriate. If ANOVA was significant, Fisher’s least significant difference test was used to determine differences among pairs of means. Early- vs. late-season hibernator and summer vs. hibernator comparisons were performed using t-tests. Some data sets [i.e., phosphorylated form of Bcl-2 (pBcl-2) and pAkt] were log-transformed due to unequal variances among the groups. A P value of ≤0.05 was considered statistically significant. For all groups analyzed, data are presented as group means ± SE.

RESULTS

Number of TUNEL-positive enterocytes increases in late-season hibernators. Representative micrographs after TUNEL staining are shown in Fig. 2. The number of TUNEL-positive enterocytes in the midvillus region of summer squirrels was relatively low, with many squirrels having no positive cells (Fig. 2A). TUNEL-positive nuclei had a condensed and darkly stained appearance (Fig. 2B). In contrast, hibernators tended to have large numbers of TUNEL-positive enterocytes, with nuclei of normal size and a diffuse staining pattern (Fig. 2, C and D). When hibernators of all winter activity states were combined, the mean number of TUNEL-positive nuclei per 25 enterocytes (8.9 ± 1.0, n = 55) was greater than in summer squirrels (1.1 ± 0.4, n = 17, P < 0.001; Fig. 3A). Further analysis using two-way ANOVA indicated that, although activity state did not influence the number of TUNEL-positive cells in hibernators (Fig. 3A), the time that squirrels had spent in hibernation had a significant effect. Late-season hibernators (≥3 mo of hibernation) had more TUNEL-positive cells than early-season hibernators (hibernating <3 mo) or summer squirrels, and there was no difference between summer and early-season hibernators (Fig. 3B). Analysis of activity records indicated that late-season hibernators underwent 14.8 ± 0.5 torpor-arousal cycles (n = 32), which was greater than early-season hibernators (8.2 ± 0.6 torpor-arousal cycles, n = 23, P < 0.001). Although we focused on epithelial cells in this study, visual inspection of the lamina propria suggested that TUNEL staining also increased in immune cells as the hibernation season progressed.

DNA ladders are not present in summer or hibernating ground squirrel mucosa. To determine whether DNA strand breaks detected by TUNEL were characteristic of apoptosis, we separated mucosal DNA by agarose gel electrophoresis to determine the extent of DNA laddering (Fig. 4). The positive control (indicated by * in Fig. 4) consisted of U937 cells...
treated with 4 µg/ml camptothecin for 3 h. No DNA ladders were observed in any squirrel group. However, considerable smearing was observed in samples from late-season hibernators (Fig. 4B), which was eliminated by RNase (but not DNase) digestion of the samples before electrophoresis (2 µg of RNase for 25 min at 37°C; Fig. 4C). The absence of DNA ladders suggested that the cells were not apoptotic, consistent with the paucity of TUNEL-positive cells with morphological characteristics of apoptosis.

**Nuclear p53 decreases during hibernation.** To determine whether the apparent increase in nonapoptotic DNA damage in late-season hibernators affected cellular signaling pathways, we examined p53 levels in nuclear extracts. p53 is a transcription factor that translocates to the nucleus when activated by DNA damage and results in either apoptosis or cell cycle arrest (38, 43). Nuclear levels of p53 decreased for all hibernators as a group and for each individual hibernating state compared with summer (Fig. 5A). When individual hibernation states were combined, late-season hibernators had greater amounts of nuclear p53 than early-season hibernators, although both were less than summer squirrels (Fig. 5C).

**Expression of Bcl-2 family members increases during hibernation.** Representative immunoblots of Bcl-2 family proteins are shown in Fig. 6. Compared with summer squirrels, expression of the proapoptotic protein Bax was about twofold greater for all hibernators as a group and for each hibernation state (Fig. 7A). Expression of the antiapoptotic protein Bcl-xL showed a similar pattern, except that the increase during hibernation was ~12-fold greater compared with summer squirrels and the value in entrance hibernators was not different from summer (Fig. 7B). There were no differences in expression of the antiapoptotic protein Bcl-2 among any ground squirrel groups (Fig. 7C), but pBcl-2 increased in mucosa of hibernating squirrels when all winter activity states were combined (Fig. 7D). All winter groups except the interbout arousal squirrels expressed higher levels of pBcl-2 compared with summer squirrels. There were no differences between early- and late-season hibernators in expression of any of the Bcl-2 family members studied (data not shown).

**Expression of active caspase-8 and -9 during hibernation.** Expression of active caspase-9 did not vary from summer to hibernating states (data not shown). However, its expression was greater in early-season compared with late-season hibernators and with summer squirrels (Fig. 8A, representative blot in Fig. 6). Despite a trend for increased levels of the active form of caspase-8 in some hibernation states, there were no differences among any ground squirrel groups (Fig. 8B, representative blot in Fig. 6) or between early- and late-season hibernators (data not shown).

**Caspase-3 activity is lower during interbout arousal compared with summer.** To determine whether hibernation affected the activity of caspase-3, we analyzed whole mucosal lysates from eight summer and eight interbout arousal squirrels using a specific caspase-3 activity assay. Caspase-3 activity was significantly lower in interbout arousal squirrels compared with summer squirrels (Fig. 9). There were no differences between early- and late-season hibernators (data not shown).

**Expression of Akt increases during hibernation.** Expression of the pro-survival protein Akt increased in intestinal mucosa during hibernation (Fig. 10B). Because Akt is activated by phosphorylation, we examined pAkt in the same samples. pAkt levels were similar in summer squirrels and hibernators when all winter activity states were combined (Fig. 10C). However, there were differences among the six squirrel groups as assessed by ANOVA. Mucosal samples from the early torpor and late torpor states expressed the least pAkt, whereas the entrance, arousal, and interbout arousal states were not significantly different from summer. Early- and late-season hibernators had similar levels of Akt and pAkt (data not shown).

**DISCUSSION**

Apoptosis is a highly conserved process in multicellular organisms that plays important roles in development, normal tissue function, and disease pathogenesis (15, 24). In enterocytes, which undergo a high rate of turnover (3–6 days in most adult mammals), some level of apoptosis is required to eliminate defective cells and to control cell numbers to maintain tissue homeostasis (24). A spontaneous level of apoptosis occurs in crypt stem cells, with up to 10% undergoing apoptosis under normal conditions. It has been suggested that enterocytes are extruded from villus tips as a consequence of apoptosis, although this idea has been questioned (24). Enterocyte apoptosis along the crypt-villus axis is also associated with a variety of stress conditions, including fasting (23), total parental nutrition (14), and intestinal ischemia-

**Fig. 3.** Number of TUNEL-positive enterocytes in midvillus regions. A: comparison of summer squirrels (SUM) (n = 17) and 5 hibernation states (early- and late-season hibernators combined for each state; n = 10–12 per group). *SUM significantly different from all hibernators combined and from each hibernation state (P < 0.001). B: TUNEL-positive cells in SUM (n = 17), all early-season hibernators (n = 23), and all late-season hibernators (n = 32) (individual hibernation states combined). *Late-season hibernators significantly greater than SUM or early-season hibernators (P < 0.001).
reperfusion (37). In contrast, few studies have examined enterocyte apoptosis under natural conditions that significantly alter the intestinal milieu. One such state is hibernation, which is characterized by extended fasting and major changes in metabolism and blood flow in the gastrointestinal tract. Although previous work from our laboratory and others documented the marked changes in enterocyte proliferation that occur during the hibernation season (10, 27), there is little information on how hibernation affects rates of enterocyte apoptosis (or apoptosis of any other cell type).

We used TUNEL to examine apoptosis in jejunum of summer and hibernating squirrels. This technique detects broken 3’ overhang DNA molecules that typically appear during the late stages of apoptosis (30). Similar to the pattern in other mammals, TUNEL-positive enterocytes in summer squirrels were rare and found primarily in the crypts. These cells displayed shrunken morphology with condensed and darkly stained nuclei, typical of apoptotic cells. The pattern differed dramatically from late-season hibernators, which displayed large numbers of TUNEL-positive enterocytes that were of normal size with typical oval nuclei. Staining tended to be diffuse and darker around the nuclear membrane. Although diffuse TUNEL staining in the midvillus region was also observed in nonhibernating species under stress states such as fasting and ischemia-reperfusion, in these studies, tissues also displayed DNA ladders (23, 37). Interestingly, the frequency of TUNEL-positive cells in early-season hibernators was similar to that of summer squirrels; however, the staining pattern resembled that of late-season animals. Thus the late-season pattern appears to accumulate as the hibernation season progresses.

TUNEL staining is not necessarily representative of apoptosis. For example, positive TUNEL staining has been associated with gene transcription (26), DNA repair (25), and nonapoptotic DNA damage (30). One way to distinguish apoptosis from nonapoptotic DNA damage is to separate DNA by agarose gel electrophoresis. A characteristic laddering pattern results from internucleosomal DNA cleavage by apoptosis-specific DNases (42). Consistent with the TUNEL results, the majority of summer squirrels and early-season hibernators had intact DNA with no evidence of laddering. Although no ladders were evident in late-season hibernators, many samples appeared as smears that were eliminated by RNase treatment. The significance of this apparent RNA contamination of samples from late-season hibernators is not clear and requires further study. Nonetheless, the DNA electrophoresis results suggest that most TUNEL-positive enterocytes in late season hibernators are not apoptotic. Because TUNEL can detect nicks in DNA that electrophoresis may not reveal, it is possible that the diffuse TUNEL staining in many enterocytes of late-season hibernators represents nonapoptotic DNA damage.

DNA damage induces the expression and nuclear translocation of the transcription factor p53 (43, 51). Once activated, p53 can induce cell cycle arrest or apoptosis, depending on the extent of DNA damage and the levels of survival factors (38, 43). Hibernators had fourfold less nuclear p53 in intestinal

Fig. 4. Representative agarose gels of mucosal DNA of summer squirrels, early-season hibernators (A), and late-season hibernators (B and C). Note increased smearing in late-season hibernators (B), which is eliminated by RNase digestion (C), leaving a pattern similar to summer squirrels and early-season hibernators. **∗** Positive control for apoptosis. L, bench top ladder.
mucosa than did summer squirrels. However, within the hibernation season, nuclear p53 was significantly greater in late-season vs. early-season hibernators, consistent with increased TUNEL staining. Thus lower levels of nuclear p53 during hibernation may reflect a compensatory response that suppresses enterocyte apoptosis despite accumulation of DNA damage as hibernation progresses.

We examined expression of several Bcl-2 family proteins that are involved in the intrinsic pathway of apoptosis. This is a well-conserved family that includes proapoptotic (e.g., Bax, Bak) and antiapoptotic (e.g., Bcl-2, Bcl-xL) members (15, 42). The relative expression of pro- to antiapoptotic Bcl-2 family members determines whether a cell will die via apoptosis (15). Expression of Bax and Bcl-xL were both elevated in intestinal mucosa of hibernators compared with summer animals. However, the greater increase in Bcl-xL expression (12-fold) relative to that of Bax (2-fold) suggests proapoptotic influences during hibernation that are countered by increased expression of Bcl-xL, resulting in suppression, rather than activation, of the intrinsic apoptotic pathway.

Although expression of another antiapoptotic protein, Bcl-2, tended to be high in some hibernator groups, there were no differences between summer and hibernating squirrels. However, pBcl-2 increased during hibernation, particularly in the torpid and arousing states. The physiological relevance of Bcl-2 phosphorylation is still under investigation. The antiapoptotic action of Bcl-2 was shown to be disabled by phosphorylation (36, 39), although this was identified primarily in response to chemotherapeutic agents. Other studies suggest that Bcl-2 phosphorylation enhances its antiapoptotic actions (16, 22, 46, 50). Interestingly, pBcl-2 appears in cells that are in G2/M-phase arrest (31, 36, 46), which may be relevant to this study. The increased levels of pBcl-2 that we observed in mucosa of torpid (but not aroused) squirrels may reflect G2/M arrest because previous studies suggest that crypt cells are blocked at this stage during torpor (27). However, because Bcl-2 phosphorylation has not been studied in enterocytes, its role in the intestine during hibernation remains to be clarified.

Anti-apoptotic Bcl-2 proteins can prevent disruption of the outer mitochondrial membrane and thus inhibit cytochrome c release from the intermembrane space (15). The presence of cytochrome c in the cytoplasm can activate caspase-9 (34, 42).
Although expression of active caspase-9 was similar in summer squirrels and hibernators (data not shown), early-season hibernators expressed more active caspase-9 compared with late-season animals. The reason for this difference is unclear, but one possibility is a preconditioning effect associated with torpor-arousal cycles (7). That is, events occurring early in the season (e.g., changes in splanchnic blood flow) may exert moderate stress, sufficient to activate caspase-9 but not apoptosis and promote tissue survival during the remainder of the hibernation season. This scenario is supported by other studies showing that ischemic preconditioning can reduce subsequent ischemia-reperfusion-induced apoptosis in the intestinal mucosa via inhibition of the intrinsic pathway (47).

We also examined the effect of hibernation on the extrinsic pathway of apoptosis, which is activated when death signals such as TNF-α or Fas ligand bind to their respective receptors and activate caspase-8 (15, 42). An increase in mucosal immune cells during hibernation (8, 18, 40) could be one mechanism for enhanced death signaling. However, despite a trend for increased active caspase-8 in the mucosa during hibernation, there was no significant difference between summer and hibernating squirrels. Thus, as for the intrinsic pathway, we...
found little evidence for activation of the extrinsic apoptotic pathway during hibernation.

Cleavage of DNA and cytoskeletal proteins following caspase-3 activation is a final stage in both the intrinsic and extrinsic apoptotic pathways subsequent to activation of caspases-9 or -8 (15, 42). We measured caspase-3 activity in mucosa from summer and interbout arousal squirrels. The latter were chosen because their $T_b$ (37°C) is similar to that of summer squirrels, and, because the assay was run at 37°C, the results should be representative of enzyme activity in vivo for both groups. The reduced activity of caspase-3 in interbout arousal squirrels suggests a specific suppression during hibernation of this important effector of apoptosis. This could occur, for example, via increased expression of inhibitor of apoptosis proteins, which can bind to and inhibit caspase-3 (52).

Expression of the prosurvival protein Akt in intestinal mucosa is also altered by hibernation. Akt is a serine-threonine kinase whose activation is typically induced by a growth factor stimulus and is mediated by phosphatidylinositol 3-OH kinase signaling (45). We observed a nearly 20-fold increase in the expression of Akt in hibernating squirrels compared with summer animals. Akt has antiapoptotic effects in a variety of cell types, including enterocytes (19, 48). It can inhibit proapoptotic actions of Bax (45), inactivate caspase-9, and activate other antiapoptotic pathways, such as NF-κB signaling (32, 45). Akt also promotes the translocation of Mdm2, a ubiquitin ligase, to the nucleus where it targets p53 for degradation by the proteasome (51). Thus increased Akt in intestinal mucosa may contribute to the decreased nuclear p53 levels observed in hibernators.

The specific induction of Akt expression in response to a stimulus or naturally occurring event is unusual; increased phosphorylation of the protein, which is thought to mediate its prosurvival activity (32, 35), is more commonly observed. pAkt levels were lower in the torpid states (early and late torpor) compared with summer, although other hibernation states were unchanged. This pattern suggests that although Akt expression is elevated during hibernation, a substantial pool of unactivated protein is maintained. This may facilitate rapid activation during interbout arousals, when release of growth factors in the intestinal mucosa and subsequent phosphorylation are most likely. Interestingly, rapid phosphorylation of Akt during arousal from torpor has been reported in the brain of hibernating bats (29). A different study reported decreased Akt and pAkt in brain, kidney, liver, and white adipose tissue and an increase in brown adipose tissue of torpid bats compared with fully aroused animals (17). Reduced levels of pAkt and no change in total Akt expression were reported in brain, muscle, liver, heart, and kidney of torpid 13-lined ground squirrels compared with active squirrels that did not hibernate during winter (3). In marmots, Akt expression in white adipose tissue was similar in summer and winter states, although activity was greater in summer animals compared with aroused hibernators (21).

**Fig. 9.** Specific activity of caspase-3 in intestinal mucosa of summer and IBA squirrels ($n = 8$ per group). *$P < 0.01$.

**Fig. 10.** Expression of total Akt and phosphorylated form of Akt (pAkt) in intestinal mucosa. A: representative immunoblots. B: densitometric analysis of Akt bands in SUM ($n = 9$) and in each hibernation state ($n = 5–9$ per group). *SUM significantly different from all hibernators combined ($P < 0.001$) and from each hibernation state ($P < 0.05$). C: pAkt expression in SUM ($n = 9$) and in each hibernation state ($n = 5–9$ per group). Groups with different letters are significantly different. There was no significant difference between SUM and all hibernators combined.
What features of the hibernation phenotype might favor apoptosis in intestinal cells and thus stimulate the anti-apoptotic mechanisms proposed here? Apart from mucosal atrophy, there is minimal damage or dysfunction during hibernation (4). Yet, the hibernator intestine sustains some level of oxidative stress, as indicated by changes in glutathione redox balance (11), increased lipid peroxidation (9), and expression of stress proteins (11, 12). Many of the physiological changes during the hibernation season share similarities with conditions that increase oxidative stress in nonhibernating species, such as fasting (23), total parental nutrition (14), and intestinal ischemia-reperfusion (37). Cellular senescence may also contribute to oxidative stress in the hibernator gut, since enterocyte proliferation and migration are minimal during torpor and resume on arousal. Cells can remain on villi for up to several weeks, much longer than the 3–6 days typical of most mammals (5, 10). Cellular senescence is associated with reduced stress tolerance (44), accumulation of oxidatively damaged macromolecules (28, 41), and increased apoptosis (49).

We showed previously that nuclear translocation of NF-κB is increased in intestinal mucosa during hibernation (9), a finding that may be relevant to the present results. NF-κB is involved in many cellular processes, including the response to oxidative stress and the regulation of apoptosis (1). NF-κB transcriptional activity may modulate expression of several of the proteins that we studied here, including Bcl-xL (13) and Akt (35). NF-κB also suppresses p53 through upregulation of Mdm2 (43). Activation of NF-κB thus appears to be an important survival strategy employed by intestinal epithelial cells during hibernation.

In conclusion, our results suggest that, despite factors that tend to promote a pro-oxidative, proapoptotic environment in the intestine during hibernation and the evidence for accumulation of DNA damage, cellular mechanisms of apoptosis are suppressed and tissue necrosis is minimized. The maintenance of intestinal integrity during hibernation is likely a key adaptation that enables the rapid restoration of body mass and protein when feeding resumes in the spring. It will be interesting to address in future studies whether patterns similar to those shown here for the intestine also occur in other tissues of hibernating mammals.

ACKNOWLEDGMENTS

We thank Mike Grahn, Jessica Reimer, Katie Luterbach, and Melissa Lefton for technical expertise; Dr. Murray Clayton for statistical advice; Dr. Sandy Martin for comments on the manuscript; and Dr. Gregory Florant for early assistance with Akt studies.

GRANTS

This work was supported by Army Research Office Grant DAAD190110455.

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