Enhanced colon tumor induction in uncoupling protein-2 deficient mice is associated with NF-κB activation and oxidative stress

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Oxidative stress has a complex effect on cancer development. To further study this process, we induced colon tumors with azoxymethane (AOM) in mice deficient for uncoupling protein-2 (UCP2). UCP2 has recently emerged as a negative regulator of mitochondrial oxidative production. When overexpressed, UCP2 protects cells from oxidative stress, while its absence may cause abundance of reactive oxygen species, release of pro-inflammatory cytokines and persistent activation of nuclear factor kappaB (NF-κB), a pleiotropic transcription factor with an increasingly recognized role in cancer. Here we show that Ucp2−/− mice develop more aberrant crypt foci and colon tumors than Ucp2+/+ littermates when examined 24 weeks after the completion of treatment with AOM (10 mg/kg i.p. weekly for a total of 6 weeks, n = 8–12). This effect is primarily seen in the proximal colon of Ucp2−/− mice (P < 0.05), in association with changes indicative of increased oxidative stress (increased staining for malondialdehyde and inducible nitric oxide synthase), enhanced NF-κB activation (increased levels of phosphorylated IκB and increased nuclear presence of p65) and a disrupted balance between intestinal epithelial cell proliferation (greater 5-bromo-2-deoxy-uridine incorporation rates and increased phosphorylation of ERK1/2 and AKT) and apoptosis (decreased number of terminal deoxynucleotidyltransferase-mediated nick-end-labeling (TUNEL)-positive cells and increased expression of Bcl-2). In conclusion, our findings provide the first in vivo evidence for a link between UCP2 and tumorigenesis and indicate the need for additional studies to assess the role of mitochondrial uncoupling in cancer development.

Introduction

Cancer development is nurtured by the sustained relationship of two pathologic processes, oxidative stress and chronic inflammation (1,2). Reactive oxygen species (ROS), a group of free oxygen radicals and non-radical compounds continuously generated during aerobic life, form the basis of oxidative stress. Akin to a dose–response effect, ROS elicit a wide spectrum of cellular responses ranging from cell proliferation to temporary or permanent growth arrest to apoptotic or necrotic cell death (3,4). Chronic inflammation is characterized by the release of cytokines or other mediators and activation of inflammatory signaling pathways (5). A central player in these events is nuclear factor kappaB (NF-κB), a transcription factor that regulates a number of genes including those involved in inducing inflammation and oxidative stress, promoting cell growth and inhibiting apoptosis (6). Activation of NF-κB demonstrates the complex interplay of oxidative stress and inflammation since it may respond to, not only promote, intracellular ROS production (7,8).

Mitochondria are a major source of intracellular ROS production (9,10). These organelles carry out oxidative phosphorylation during which substrate oxidation by the electron transport chain builds a proton gradient across the inner membrane and fuels ATP synthesis. Superoxide, a key ROS radical, is produced during this process by incomplete reduction of molecular oxygen (9,10). Regarded as a harmful spin-off, this pathway normally involves <1% of the transported electrons (11), but escalates when mitochondrial membrane potential (ΔΨm) is high and there is an electron glut due to substrate excess or impaired oxidative phosphorylation machinery (9,10). Importantly, even a modest increase in proton leak (i.e. reentry of protons into the mitochondrial matrix without driving ATP synthesis) may readily diminish ΔΨm and the formation of superoxide, indicating that ‘mild uncoupling’ is a powerful tool to reduce...
oxidative stress (12–14). Hence, interest is rising in mitochondrial uncoupling proteins as potential agents to control ROS production, with uncoupling protein-2 (UCP2) as a leading candidate due to its wide tissue distribution (14,15).

Significant evidence indicates that UCP2 functions as a sensor and negative regulator of mitochondrial ROS production. Thus, overexpression of UCP2 provides protection from oxidative stress in mouse cardiomyocytes (16), neuronal cells (17), macrophages (18) and human THP1 monocytes (19). Moreover, genetic ablation or pharmacologic inhibition of UCP2 produces more ROS in macrophages (18,20), endothelial cells (21), hepatocytes (22) and pancreatic beta-cells (23). Recent work indicates that UCP2 functions as a sensor and negative regulator of mitochondrial ROS production. Thus, overexpression of UCP2 provides protection from oxidative stress in mouse cardiomyocytes (16), neuronal cells (17), macrophages (18) and human THP1 monocytes (19).

Moreover, genetic ablation or pharmacologic inhibition of UCP2 produces more ROS in macrophages (18,20), endothelial cells (21), hepatocytes (22) and pancreatic beta-cells (23). Recent work indicates that UCP2 functions as a sensor and negative regulator of mitochondrial ROS production. Thus, overexpression of UCP2 provides protection from oxidative stress in mouse cardiomyocytes (16), neuronal cells (17), macrophages (18) and human THP1 monocytes (19).

Because baseline UCP2 is significant in the gastrointestinal tract (27), we chose a model of colon tumorigenesis to critically analyze the role of UCP2 in cancer development. We treated Ucp2<sup>−/−</sup> mice with azoxymethane (AOM), an experimental alkylating carcinogen initiating aberrant crypt foci (ACF) that may progress into cancer with time (28). This progression is modulated by oxidative stress and inflammation (29,30). Advanced AOM-induced lesions primarily occur in the distal colon and exhibit genetic and pathologic features that resemble the sporadic form of human colon cancer (31,32). We show that Ucp2<sup>−/−</sup> mice are increasingly susceptible to the carcinogenic effect of AOM. In addition, enhanced ACF and colon tumor formation in Ucp2<sup>−/−</sup> mice is associated with increased oxidative stress, NF-κB activation and a disrupted balance between intestinal epithelial cell proliferation and apoptosis. These findings provide the first <i>in vivo</i> evidence for a link between mitochondrial uncoupling proteins and cancer. Intriguingly, enhanced tumorigenesis in Ucp2<sup>−/−</sup> mice primarily affects the proximal colon, raising the speculation that UCP2 deficiency provides a model for human proximal colon cancer associated with epigenetic events.

**Materials and methods**

**Reagents**

AOM was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO) and freshly dissolved in 0.9% sterile saline at a concentration of 1 mg/ml, 1 h prior to injection. All other chemicals were from Sigma unless otherwise specified.

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**Fig. 1.** AOM-induced colon lesions in Ucp2<sup>−/−</sup> mice. (A) Stereomicroscopic views of methylene blue-stained proximal colon segments of Ucp2<sup>−/−</sup> mice 24 weeks following the completion of treatment with AOM show two small ACF consisting of one and two small crypts (upper panel, arrows) and a large ACF with a cluster of at least six aberrant crypts (lower panel, arrowheads). Scale bar, 100 μm. (B) Macroscopic view of a longitudinally opened Ucp2<sup>−/−</sup> mouse colon with tumors in the proximal (arrowhead) and distal segment (arrow). Scale bar, 10 mm. (C) H&E-stained tissue sections of a distal colon tumor identified as adenoma. Scale bars, 1 mm (left panel) and 100 μm (right panel). (D) Individual scores of AOM-induced large ACF. (E) Colon tumors and (F) largest tumor diameters in Ucp2<sup>−/−</sup> mice and Ucp2<sup>+/+</sup> littermates. Prox, proximal colon; dist, distal colon. Horizontal lines indicate median scores. Histological evaluation indicated that all tumors were adenomas. Large ACF and colon tumors were absent in all saline-treated control animals (data not shown). Asterisk (*), P < 0.05 between Ucp2<sup>−/−</sup> and Ucp2<sup>+/+</sup> mice; double plus (‡), P < 0.05, pair of double plus (‡‡), P < 0.01 between proximal and distal colon.
**Results and discussion**

Twenty-four weeks after the completion of AOM treatment, mice of both genotypes had similarly lower body weight when compared with saline-treated controls (Ucp2<sup>+/−</sup> mice, 26.1 ± 0.8 versus 32.9 ± 1.9 g; *P* < 0.05; Ucp2<sup>+/+</sup> mice, 26.6 ± 0.9 versus 31.1 ± 2.1 g; *P* < 0.05). Stereomicroscopy revealed numerous ACF (>30/mouse) in the colon of Ucp2<sup>+/−</sup> mice and Ucp2<sup>+/+</sup> littermates. However, most of these ACF consisted of 1–3 small crypts, with only a few lesions that contained >4 crypts (large ACF) (Figure 1A and D), supporting the notion that many ACF fail to advance (34). As expected, development of large ACF was limited to the distal colon in Ucp2<sup>+/−</sup> mice. In Ucp2<sup>+/−</sup> mice, however, large ACF also developed in the proximal colon (P = 0.029 versus Ucp2<sup>+/+</sup> mice). The distribution of large ACF was mirrored by colon tumors (Figure 1B and C). Thus, tumors developed in the distal colon regardless of the genotype, although the tumor size was larger in Ucp2<sup>+/−</sup> mice than in Ucp2<sup>+/+</sup> mice (largest diameter, 3.0 ± 1.3 versus 1.25 ± 0.8 mm, NS) (Figure E and F). In the proximal colon, tumors developed only in Ucp2<sup>+/−</sup> mice.

Fig. 2. Cell proliferation and apoptosis in the colon of AOM-treated Ucp2<sup>+/−</sup> mice. (A) Intestinal epithelial cell proliferation rates were assessed by nuclear immunostaining for BrdU and (B) apoptosis rates were assessed by TUNEL assay and given as cumulative scores from 20 colonic crypts of the genotype, although the tumor size was larger in Ucp2<sup>+/−</sup> mice than in Ucp2<sup>+/+</sup> mice (largest diameter, 3.0 ± 1.3 versus 1.25 ± 0.8 mm, NS) (Figure E and F). In the proximal colon, tumors developed only in Ucp2<sup>+/−</sup> mice.
mice at an average diameter of 1.0 ± 0.4 mm), whereas this segment remained tumor-free in Ucp2+/+ mice (P = 0.025 versus Ucp2−/− mice) (Figure E and F). These findings indicate an increased susceptibility to AOM in the proximal colon of Ucp2−/− mice.

To further assess the impact of UCP2 deficiency on AOM-induced colon tumorigenesis, we assessed various markers of proliferation and apoptosis (Figure 2). We measured proliferation rates of intestinal epithelial cells in AOM-treated animals by BrdU incorporation and found that the number of cells with BrdU-positive nuclei was 80% higher in the proximal colon of Ucp2−/− mice when compared with Ucp2+/+ mice (Figure 2A). Since phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) is linked to pathways that activate cell growth and proliferation, we determined the level of pERK1/2 and found it higher in both colon segments of AOM-treated Ucp2−/− mice (Figure 2C). In addition, amounts of phosphorylated AKT, another effector of cell survival and proliferation, were highest in the proximal colon of Ucp2−/− mice (Figure 2E). These findings indicate the presence of enhanced cell survival and proliferation signals that

![Graph showing relative expression of pIkB and β-actin in proximal (prox) and distal (dist) colon of Ucp2+/+ and Ucp2−/− mice.](image)

![Image with immunohistochemistry results showing nuclear presence of p65 in intestinal epithelial cells of AOM-treated mice.](image)

![Image with immunohistochemistry results showing presence of MDA and iNOS in intestinal epithelial cells.](image)

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**Fig. 3.** NF-κB activation and oxidative stress in the colon of AOM-treated Ucp2−/− mice. (A) Phosphorylated IkB (pIkB) levels were assessed by western blot analysis in the colon of Ucp2−/− mice and Ucp2+/+ littermates 24 weeks following the completion of treatment with AOM. Fold changes in densitometry values ± SEM are expressed relative to the proximal colon of AOM-treated Ucp2+/+ mice. Representative western blots are shown from at least three independent experiments (with two lanes representing each treatment group and showing β-actin as a loading control). Prox, proximal colon; dist, distal colon. (B) Nuclear presence of p65 in intestinal epithelial cells of AOM-treated mice was assessed by immunohistochemistry. Scores of p65-positive nuclei averaged from 20 colonic crypts and (C) representative colon tissue sections stained for p65 (scale bar, 100 μm) are shown. (D) Presence of cytoplasmic MDA and (E) iNOS in intestinal epithelial cells was assessed by immunohistochemistry. IOD values obtained from the lower third of the colonic crypts are expressed in arbitrary units ± SEM. (F) Representative tissue sections of MDA-stained crypts (scale bar, 100 μm). Asterisk (*), P < 0.05 and pair of asterisks (**), P < 0.01 between Ucp2−/− and Ucp2+/+ mice. Double plus (‡), P < 0.05 between proximal and distal colon.
correlate with tumor formation in the proximal colon of AOM-treated Ucp2−/− mice.

Apoptosis among intestinal epithelial cells was diminished in the proximal colon of AOM-treated Ucp2−/− mice (Figure 2B), suggesting that insufficient apoptosis also contributes to increased tumorigenesis in the absence of UCP2. To better characterize the apoptotic response, we examined the colonic expression of pro-apoptotic (Bak and Bax-α) and anti-apoptotic (Bcl-2 and Bcl-Xl) members of the Bcl-2 protein family in AOM-treated mice. We found that UCP2 deficiency had no significant impact on colon tissue protein levels of Bak (Figure 2D) and Bax-α (data not shown). In contrast, Bcl-2 expression was almost undetectable in the proximal colon of Ucp2−/+ mice, while its expression in Ucp2−/− mice was considerable when compared with higher Bcl-2 levels detected in the distal colon (Figure 2F). Bcl-Xl followed the segmental pattern of Bcl-2 expression (data not shown). The findings in Ucp2−/+ mice suggest that low Bcl-2 levels contribute to higher apoptotic rates in the proximal colon, a location repeatedly found to be more resistant to chemical induction of cancer (35,36). At the same time, perturbed regulation of anti-apoptotic Bcl-2 proteins in the proximal colon of Ucp2−/− mice may have an important role in AOM-induced tumorigenesis.

Because substantial evidence implicates the role of NF-κB in cancer by inducing genes that promote cell proliferation and inhibit apoptosis (6) and UCP2 deficiency promotes the activation of this critically important transcription factor (37), we analyzed NF-κB activation in the colon of AOM-treated Ucp2−/+ mice. In the canonical NF-κB pathway, phosphorylation of the inhibitory IκB protein by its upstream kinase (IKKβ) allows the release and nuclear translocation of p65/p50 heterodimers to induce transcription of genes critical to the regulation of inflammation, cell growth and apoptosis (6). To assess IKKβ activation, we detected pIKKβ by western blot analysis and found it more abundant in the colon of AOM-treated Ucp2−/+ mice when compared with Ucp2+/+ littermates, indicating that NF-κB activation is augmented in the absence of UCP2 (Figure 3A). This difference was particularly apparent in the proximal colon. We also assessed NF-κB activation by detecting nuclear p65 in intestinal epithelial cells of AOM-treated animals (Figure 3B and C). The number of p65-positive nuclei was higher in the proximal colon of Ucp2−/− mice than in Ucp2+/+ littermates, indicating increased nuclear translocation of NF-κB and correlating with regional differences in AOM-induced colon tumorigenesis.

Since the presence of free radicals is often critical in cancer development (1) and increased ROS formation has been observed in UCP2 deficiency (20,22,37), we also assessed the level of oxidative stress in the colon of Ucp2−/− mice. Immunostaining for MDA, a lipid peroxidation end product utilized to evaluate oxidative injury, was higher in the distal colon of untreated mice regardless of genotypes, supporting the importance of regional differences (Figure 3D and E). Notably, MDA staining was more intense in the proximal colon of AOM-treated Ucp2−/− mice compared with Ucp2+/+ littermates.

Our study provides the first in vivo evidence for a link between UCP2 and cancer. While there is a growing appreciation that ROS control is a major function of UCP2 (14,15), the phenotype of Ucp2−/− mice remains relatively unremarkable unless these animals are challenged by metabolic stress, infection or surgery (20,22,33). Nevertheless, subtle but cumulative changes of oxidative injury and inflammation may take place in Ucp2−/− mice due to the lack of ‘mild uncoupling’. It is currently unknown if UCP2 deficiency promotes spontaneous tumor incidence, although limited data on Ucp2−/− mice aged over 2 years indicate that this might indeed be the case (15). Our findings are particularly relevant to conditions in which impaired UCP2 function may contribute to oxidative stress. Interestingly, despite the nearly ubiquitous presence of UCP2, negative regulation of its expression appears unique to macrophages (18), which may in turn produce excess ROS and display persistent NF-κB activation (20,37). Although a pathogenic role of UCP2 deficiency in human disease has not yet been established, macrophages of mice with genetic obesity exhibit diminished UCP2 expression (38). Cell-specific deletion of IKKβ in macrophages has recently demonstrated that these cells are instrumental in colon cancer development (30). Whether macrophages play such a role in Ucp2−/− mice remains to be seen.

Another notable finding of our study on Ucp2−/− mice is the predilection for enhanced tumorigenesis in the proximal colon. Cancer development in the human proximal colon is typically characterized by DNA hypermethylation and microsatellite instability (39) and ROS may promote this process (40). Additional studies will establish the usefulness of Ucp2−/− mice in modeling human colon cancer related to epigenetic changes. Although other cancer paradigms will be needed to confirm our observations, we find it tempting to speculate that altered UCP2 expression, oxidative stress and NF-κB activation are related and successive events in cancer development.

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