Ketogenic Diets Enhance Oxidative Stress and Radio-Chemo-Therapy Responses in Lung Cancer Xenografts

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Abstract

Purpose: Ketogenic diets are high in fat and low in carbohydrates as well as protein which forces cells to rely on lipid oxidation and mitochondrial respiration rather than glycolysis for energy metabolism. Cancer cells (relative to normal cells) are believed to exist in a state of chronic oxidative stress mediated by mitochondrial metabolism. The current study tests the hypothesis that ketogenic diets enhance radiotheraphy responses in lung cancer xenografts by enhancing oxidative stress.

Experimental Design: Mice bearing NCI-H292 and A549 lung cancer xenografts were fed a ketogenic diet (KetoCal 4:1 fats:carbohydrates) and treated with either conventionally fractionated (1.8–2 Gy) or hypofractionated (6 Gy) radiation as well as conventionally fractionated radiation combined with carboplatin. Mice weights and tumor size were monitored. Tumors were assessed for immunoreactive 4-hydroxy-2-nonenal-(4HNE)–modified proteins as a marker of oxidative stress as well as proliferating cell nuclear antigen (PCNA) and γH2AX as indices of proliferation and DNA damage, respectively.

Results: The ketogenic diets combined with radiation resulted in slower tumor growth in both NCI-H292 and A549 xenografts (P < 0.05), relative to radiation alone. The ketogenic diet also slowed tumor growth when combined with carboplatin and radiation, relative to control. Tumors from animals fed a ketogenic diet in combination with radiation showed increases in oxidative damage mediated by lipid peroxidation as determined by 4HNE-modified proteins as well as decreased proliferation as assessed by decreased immunoreactive PCNA.

Conclusions: These results show that a ketogenic diet enhances radio-chemo-therapy responses in lung cancer xenografts by a mechanism that may involve increased oxidative stress. Clin Cancer Res; 19(14): 3905–13. ©2013 AACR.
Ketogenic diets are high in fat, low in carbohydrates, and are well established as an alternative therapy for childhood epilepsy. This report shows that a ketogenic diet enhances radio-chemo-therapy responses as well as enhancing oxidative stress in human lung cancer xenografts. As ketogenic diets are an established therapy in humans, these studies may be rapidly translated into the clinical setting, potentially allowing for improved cancer control without added normal tissue toxicity.

Translational Relevance
Ketogenic diets are high in fat, low in carbohydrates, and are well established as an alternative therapy for childhood epilepsy. This report shows that a ketogenic diet enhances radio-chemo-therapy responses as well as enhancing oxidative stress in human lung cancer xenografts. As ketogenic diets are an established therapy in humans, these studies may be rapidly translated into the clinical setting, potentially allowing for improved cancer control without added normal tissue toxicity.

Materials and Methods
Cell culture conditions
A549 and H292 cells were obtained from the American Type Culture Collection. A549 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS (Hyclone) and 0.01% gentamycin sulfate (Cellgro). H292 cells were maintained in RPMI containing 10% FBS (Hyclone) and 0.01% gentamycin sulfate (Cellgro). Glucose-depleted media consisted of DMEM devoid of glucose and pyruvate but containing glutamine (Gibco) to which 70 mg/dl d-glucose was added.

Clonogenic survival analysis
A549 or H292 cells (120,000/60 mm dish) were plated and allowed to grow in their respective tissue culture media for 24 hours. All plates were then placed into DMEM glucose-depleted media with 70 mg/dl glucose added back. Selected tissue culture dishes were supplemented with 3 mmol/L (R)-(3-hydroxybutyric acid sodium salt (BHB; Sigma Aldrich) and 1.5 mmol/L lithium AA (Sigma Aldrich) mimicking the maximum concentration and approximate ratio of ketones found in the blood of adult humans on a ketogenic diet (14). After 24 hours of ketone treatment, cultures were exposed to sham, 0.5 Gy, 1 Gy, 2 Gy, 3 Gy, or 4 Gy ionizing radiation after which clonogenic survival analysis was conducted as previously described (15). The results show that ketogenic diet enhances radio-chemo-therapy responses as well as inducing oxidative stress in tumors and improves responses to radio-chemo-therapy in mice with lung cancer xenografts by a mechanism that seems to involve oxidative stress. Furthermore, ketogenic diets effectively enhance tumor growth inhibition and prolong animal survival in response to radio-chemo-therapy in mice with lung cancer xenografts by a mechanism that involves oxidative stress in tumor tissues.

Tumor xenograft growth
Female 4 to 6-week-old athymic-nu/nu mice were purchased from Harlan Laboratories. Mice were housed in the Animal Care Facility at the University of Iowa (Iowa City, IA) and all procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and conformed to NIH guidelines. H292 and A549 tumor cells were injected subcutaneously into the right flanks as previously described (15). Treatment began when tumor volumes measured approximately 30 mm³.

Ketogenic diet and ketone monitoring
The mice in the ketogenic diet groups were fed ad libitum KetoCal (Nutricia North America, a formula designed for children with epilepsy). This diet has a ketogenic ratio (fats: proteins + carbohydrates) of 4:1 (energy distribution: fat 90%, carbohydrate 1.60%, and protein 8.40%) and the fat is derived from soybean oil (100% long chain fatty acids; 22% saturated fat, 24% monounsaturated fat, and 15% polyunsaturated fat). KetoCal was prepared as a paste on a culture-dish lid by adding water (water: KetoCal 1:2) then placed upside down in the food hopper and attached to assure the animals access (16). Control mice were fed a standard rodent diet (NIH-31 modified 25% protein, 21% fat, and 54% carbohydrate). Ketogenic diet was started 2 days before the initiation of ionizing radiation and continued for 24 hours after the last treatment. Blood BHB and glucose levels were measured using the Precision Xtra (Abbott Laboratories) machine via a tail vein stick. Previous results indicated that the average blood levels of BHB starting 3 days after beginning the ketogenic diet and continuing through the end of treatment were 1.01 ± 0.64 mmol/L for the ketogenic diet mice versus 0.18 ± 0.08 mmol/L for control mice (17).

Ionizing radiation in vivo
Mice in all treatment and sham groups were anesthetized using 87.5 mg/kg ketamine and 12.5 mg/kg xylazine mixture in the radiation facility at the University of Iowa. The mice in the ionizing radiation groups were placed in a lead box with only the right flank exposed. Radiation was delivered using a Pantak Therapax DXT 300 X-ray machine operated at 200 kVp with added filtration of 0.35 mm Cu + 1.5 mm Al, resulting in a beam quality of 0.95 mm Cu. Tumors were measured daily using Vernier calipers (volume = (length × width²)/2) and euthanized when tumor length exceeded 1.5 cm in any dimension. For the 12 Gy total...
mice were divided into the following treatment groups (N represents the total number of animals per treatment group combined from 2 duplicative experiments); (i) control group (N = 10), (ii) ketogenic diet group (N = 12), (iii) ionizing radiation group (12 Gy total dose in 6 × 2 Gy fractions every other day for 2 weeks; N = 16), (iv) ionizing radiation + ketogenic diet group (N = 16), (v) carboplatin (15 mg/kg intraperitoneal once per week × 3 doses; N = 6), (vi) carboplatin + ketogenic diet (N = 5), (vii) ionizing radiation + carboplatin (N = 12), and (viii) ionizing radiation + carboplatin + ketogenic diet (N = 14). For the 61.2 Gy total dose-fractionated ionizing radiation experiment (Fig. 2) mice were divided into 4 groups; (i) Control (N = 6), (ii) ketogenic diet (N = 7), (iii) ionizing radiation (61.2 Gy in 34 doses, 1.8 Gy every other day; N = 8], and (iv) ketogenic diet + ionizing radiation (N = 7). The 4 treatment groups for the 12 Gy hypofractionation ionizing radiation experiment (Fig. 3) with 9 mice per treatment group were; (i) Control, (ii) ketogenic diet, (iii) ionizing radiation (12 Gy in 2 × 6 Gy fractions, 48 hours apart), and (iv) ketogenic diet + ionizing radiation. At the completion of
the ketogenic diet, the 3 mice with the largest tumors from each treatment group were collected and frozen in liquid nitrogen for oxidative stress assessment.

4-Hydroxy-2-nonenal-(4HNE)–modified protein immunoblotting assay

Approximately 20 mg of mouse tumor was washed and homogenized in 300 µL 500 mmol/L potassium phosphate, 50 mmol/L EDTA buffer pH 7.0 with Complete Mini-protease inhibitor (Roche Diagnostics). Protein concentrations were determined by Lowry Assay. Twenty-five micrograms of protein was placed in an equal volume with nanopure H2O and blotted onto prewetted Sequi-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and allowed to dry. After rewetting in methanol, the membrane was incubated in 250 mmol/L sodium borohydride in 100 mmol/L MOPS, pH 8.0 for 15 minutes to chemically reduce the Schiff base adduct to reveal the Michael addition product for antibody recognition. The membrane was then washed 3 times each with nanopure H2O followed by PBS, then blocked for 30 minutes in 2% albumin in PBS + 1% Tween 20. The blot was incubated with the primary antibody recognizing the Michael addition product of 4HNE-modified cellular proteins (18) diluted 1/2,000 overnight at 4°C, followed by 2 hours in secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1/20,000), and chemiluminescence detection (ECL Plus Western Blotting Detection System, GE Healthcare) with X-ray film. Immunoreactive protein on the dot blot was analyzed using integrated densities determined using ImageJ software.

Western blot assays

Approximately 100 mg piece of tumor was homogenized in 0.5 mL of ice-cold Tris buffer pH 7.8 with Complete Mini-protease inhibitor and PhosStop phosphatase inhibitor cocktail (Roche Diagnostics). After a freeze/thaw, the samples were centrifuged at 10,000 × g for 15 minutes at 4°C. Protein concentrations were determined by Lowry method. Proliferating cell nuclear antigen (PCNA) Western blot analysis was carried out using a monoclonal anti-PCNA clone PC10 antibody (M0879, Dako) running both 10 and 70 µg of protein per lane on separate occasions, electrophoresed on 4% to 20% gradient SDS-polyacrylamide gels. Bands were detected using ECL Plus Western Blotting Detection System (GE Healthcare) with detection by either X-ray film and/or a Typhoon FLA 7,000 fluorescent detection system. Band-integrated densities were determined with ImageJ software and averaged between the two gels. The γH2AX Western was conducted following the same procedure described for PCNA but running on a 15% SDS-polyacrylamide gel using reaction with the polyclonal anti-phos-H2AX Ser 139 antibody (#07-164, Upstate Biotechnology).

Statistical analysis

The analysis of in vivo results focused on treatment group comparisons of tumor growth and survival. Regression analysis was used to model tumor growth as a non-linear function of follow-up time and to make treatment group comparisons. Within-animal correlation structures were included in the models to account for repeated
measured over time. Plots of the estimated tumor means and their standard errors are provided in the results section. Estimates of survival were obtained with the methods of Kaplan–Meier and compared with log-rank tests. All associated statistical tests were 2-sided and assessed for significance at the 5% level with the SAS statistical software.

**Results**

**Ketogenic diet enhances radio-chemo-therapy responses in mouse non–small-cell lung carcinoma xenografts**

Athymic nude mice were injected with 2–3 × 10⁶ H292 non–small-cell lung carcinoma (NSCLC) cells in their right flank. When tumors reached approximately 4 mm in diameter, mice were treated with ionizing radiation alone or ionizing radiation combined with: ketogenic diets, carboplatin, and/or standard fractionated ionizing radiation doses (12 Gy in 6 × 2 Gy fractions). All animals consuming a ketogenic diet for 48 hours or more had serum ketone levels more than 0.3 mEq/L (or 0.3 mmol/L) and were considered to be in ketosis as per the Emory Warner Clinical Laboratories of the University of Iowa Hospitals and Clinics. Consistent with previous studies (17), the mice eating the ketogenic diets showed blood βHB levels more than 0.3 mEq/L (mean = 1.4 ± 0.8 mEq/L) throughout the treatment period, whereas mice treated with standard mouse chow were consistently less than 0.3 mEq/L (mean 0.1 ± 0.1 mEq/L).

Mice treated with ketogenic diet + ionizing radiation or ketogenic diet + ionizing radiation + carboplatin (12 Gy in 6 × 2 Gy fractions ionizing radiation) showed a significant decrease in tumor growth rate compared with mice treated with identical therapies on standard chow (P = 0.0470 and P = 0.0046, respectively; Fig. 1A). Furthermore, mice treated with ketogenic diet + ionizing radiation also showed a significant survival advantage over mice treated with ionizing radiation alone (P = 0.0281); survival of mice treated with ketogenic diet + ionizing radiation + carboplatin versus ionizing radiation + carboplatin was also prolonged (P = 0.0595) but did not reach less than 0.05 significance level (Fig. 1B). All treatments were well tolerated as shown by a lack of weight loss as well as general condition and activity of the mice (Fig. 1C). These data support the conclusion that ketogenic diets can effectively enhance responses to radiochemo-therapy in animals bearing NSCLC xenografts without causing overt signs of toxicity.

**Ketogenic diets enhance responses of lung cancer xenografts to hypofractionated radiation**

Hypofractionated radiation therapy (delivering higher doses in fewer fractions) has become of increasing interest with the recognition of a potential improvement in the therapeutic ratio between the tumor volume and normal anatomic structures. Clinically, hypofractionation allows for increased patient convenience and reduced costs without sacrificing efficacy and is used to treat early-stage lung cancers (19). In addition, hypofractionated radiation shortens the duration of radiation therapy which would potentially increase patient compliance with the diet.

For hypofractionated radiation experiments, H292 and A549 xenografts were grown in nude mice as in Figs. 1 and 2. Mice were fed a ketogenic diet for a total of 7 days starting 2 days before and continuing 2 days after 2 fractions of 6 Gy ionizing radiation (12 Gy total dose). Both H292 and A549 xenograft bearing animals treated with ketogenic diet in combination with hypofractionated ionizing radiation showed significantly slower tumor growth rates, relative to ionizing radiation alone (P = 0.0299 and P = 0.0054, respectively; Fig. 3A and C). H292 xenografts treated with ketogenic diet + ionizing radiation also showed a survival advantage compared with those treated with ionizing radiation alone (P = 0.0041), relative to ionizing radiation alone (Fig. 2B). These results clearly show that a prolonged ketogenic diet combined with a clinically relevant ionizing radiation dose fractionation schedule is well tolerated and enhances radiation response in NSCLC xenografts.

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condition of the mice (Supplementary Fig. S1A and S1B). These results show that a ketogenic diet enhances responses of NSCLC xenografts to hypofractionated radiotherapy.

**Ketogenic diets in combination with ionizing radiation increase immunoreactive 4HNE-modified proteins in tumor tissue**

To assess oxidative stress *in vivo*, immunoreactive 4-hydroxy-2-nonenal-(4HNE)–modified protein was analyzed in tumors as a marker of oxidative protein damage caused by lipid peroxidation. Dot blot analysis of tumor samples harvested at the end of the hypofractionated-ionizing radiation experiment shown in Fig. 3A and B showed increased 4HNE-modified protein in H292 xenografts treated with ketogenic diets + hypofractionated ionizing radiation versus hypofractionated ionizing radiation alone (*P* < 0.05; Fig. 4A and B). These data support the hypothesis that a ketogenic diet combined with hypofractionated ionizing radiation causes an increase in oxidative damage to proteins in tumor tissue resulting from lipid peroxidation-derived aldehydes.

**Ketogenic diets in combination with ionizing radiation decrease immunoreactive PCNA protein in tumor tissue following treatment with fractionated radiation**

To further probe possible mechanisms responsible for the inhibition of tumor growth seen when the ketogenic diet was combined with conventional fractionated radiation, tumors harvested at the end of treatment shown in Fig. 2 were analyzed by Western blotting for markers of proliferation (using immunoreactive proliferating nuclear antigen; PCNA) and DNA damage (using immunoreactive phosphorylated histone γH2AX; Fig. 5). Consistent with the inhibition in tumor growth and prolongation of survival seen in Fig. 2, tumors treated with ketogenic diet + ionizing radiation showed significantly lower levels of immunoreactive PCNA than mice in all other treatment groups (Fig. 5A and B). Phosphorylated histone γH2AX was significantly increased in tumors treated with radiation; however, mice treated with ketogenic diet + ionizing radiation did not show any further increase in levels of γH2AX indicating that enhanced radiation response does not seem to be related to increased levels of DNA damage in animals fed a ketogenic diet (Fig. 5C).

**Ketones in cell culture media do not significantly enhance radiation response in human lung cancer cells *in vitro***

To mimic ketosis in an *in vitro* system, exponentially growing H292 (Supplementary Fig. S2A) and A549 (Supplementary Fig. S2B) human lung cancer cells were grown in tissue-culture media containing 3 mmol/L βHB and 1.6 mmol/L AA and 70 mg/dL glucose for 24 hours. These concentrations of ketones and glucose mimic the concentrations and approximate ratios found in the blood of adult humans eating a ketogenic diet (14). Glucose was used at a rate of approximately 35 mg/dL per day depending on the cell concentration and was replenished daily to 70 mg/dL. A549 cells were capable of metabolizing βHB as shown by a decrease in βHB levels in the media during 5 days of growth (from 3 to 1.8 mmol/L). The combination of ketones with radiation exposure (0–4 Gy) did not alter clonogenic survival in either H292 or A549 cells, relative to similarly treated cells in standard media (Supplementary Fig. S2A and S2B).

**Discussion**

The current results show that a ketogenic diet is an effective adjuvant capable of enhancing radio-chemo-therapy responses in xenograft models of human NSCLC. It is also important to note that the ketogenic diet alone in our study did not result in any inhibition of tumor growth, relative to control. This finding is in agreement with 2 studies in orthotopic models of brain cancer (20, 21) where Maurer and colleagues showed no improvement in survival when animals were fed a ketogenic diet as well as Zhou and colleagues who could only show a ketogenic diet-induced
tumor growth inhibition when the diet was combined with caloric restriction. However, our findings in lung cancer xenografts differ from other investigations in prostate, gastric, and brain cancer models that show a positive therapeutic advantage using ketogenic diet as a monotherapy (22–24). These differences could be due to the different types of tumor and animal models used or the different fatty acid compositions of the ketogenic diets and control diets that were used. Our model uses a soy-based fatty acid composition which is relatively high in oleic (18:1), linoleic (18:2), and linolenic (18:3) acids with standard mouse chow as the control. Because unsaturated fatty acids are substrates for lipid peroxidation that may govern the formation of aldehydes such as 4HNE, this fatty acid composition may be influencing our results. Freedland and colleagues used a high fat, moderate high carbohydrate “Western” diet as the control diet in a prostate cancer model (23), whereas Otto and colleagues used a ketogenic diet supplemented with omega 3 fatty acids and medium-chain triglycerides (22). In addition, Stafford and colleagues used a syngeneic intracranial mouse model of glioma showing the potential role of tumor microenvironment in the biologic effects of ketogenic diets (24).

The current study also showed that ketogenic diets enhanced the responses of H292 and A549 NSCLC xenografts using 2 different radiation dosing schemes. In the conventional fractionation dosing scheme (61.2 Gy in 34 fractions with 1.8 Gy/fraction), significant increases in animal survival were achieved in the ketogenic diet + ionizing radiation group when compared with the ionizing radiation alone treatment group (Fig. 2). In a recent clinical trial, studying the effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer, it was concluded that the ketogenic diet was suitable for patients with advanced cancer and resulted in no severe side effects (25). However, it was also noted that 4 of the 16 patients stopped the diet before the end of a 7-week treatment time (25).

To shorten the time required to be maintained on a ketogenic diet, xenograft responses to hypofractionated radiotherapy were determined. Clinically, hypofractionation allows for both increased patient convenience and reduced costs without sacrificing efficacy and is frequently used to treat early-stage lung cancers (19, 26–28). Ketogenic diet combined with hypofractionated ionizing radiation significantly slowed tumor growth rate and prolonged survival, when compared with radiation alone, in
both the H292 and A549 lung cancer xenograft models (Fig. 3A and B). This finding in lung cancer is similar to a recent finding using a ketogenic diet with hypofractionated radiation (2 × 4 Gy fractions) to treat transplantable mouse gliomas (29).

Elevated ketone levels have been implicated in the generation of cellular metabolic oxidative stress. Jain and colleagues found elevated indices of lipid peroxidation in cultured human endothelial cells treated with AA (30). Stadler and colleagues showed an increase in protein oxidation and lipid peroxidation through a free radical–dependent mechanism in rat livers of animals subjected to a model of acetone induced ketosis (31). Chronic exposure to βHB was also shown to increase ROS production in cardiomyocytes (32). Finally, Milder and colleagues showed an increase in H2O2 production and lipid peroxidation in the hippocampus of rats fed a ketogenic diet (33). An excellent review addressing the effect of ketogenic diet on redox biology in neuronal tissues was also recently published (34).

It has been hypothesized that cancer cells, relative to normal cells, exist in a condition of chronic metabolic oxidative stress mediated by increased steady-state levels of \( \Delta^{2} \) and \( \Delta^{2} \text{H}_{2} \text{O}_{2} \), with a major site of prooxidant production being mitochondrial electron transport chain complexes. This increased level of ROS in cancer cells may be compensated for by increases in glucose metabolism though the pentose phosphate pathway resulting in the generation of NADPH to be used as a cofactor in hydroperoxide metabolism (3).

Because ketogenic diets force cells to rely on mitochondrial oxidative metabolism for energy production while limiting glucose availability, it would be expected that a ketotic state would exacerbate metabolic oxidative stress in cancer cells, relative to normal cells. Thus, a ketogenic diet may selectively enhance radio-chemo-responses in cancer cells when combined with standard cancer therapeutics through a mechanism involving oxidative stress.

Consistent with this hypothesis, the current studies also showed that tumors from mice treated with ketogenic diet and radiation showed significantly increased 4HNE-modified proteins (Fig. 4). This observation supports the hypothesis that ketogenic diet-induced enhancement of radiation response in vitro may involve increases in lipid peroxidation and protein damage that could contribute to growth inhibition as assayed by lower levels of immunoreactive PCNA (Fig. 5). The accumulation of 4HNE-modified proteins in tumors treated with the ketogenic diet combined with radiation are also consistent with previous results in breast cancer cells showing that exposure to a mixture of conjugated linoleic acid isomers could selectively inhibit breast cancer cell proliferation by increasing levels of 4HNE (35).

Interestingly, H292 and A549 cells exposed in vitro to ketones and ionizing radiation doses ranging from 0.5 Gy to 4 Gy did not show enhanced radiation responses. Previous research has also showed that radiation responses in vitro do not necessarily correlate with radiation responses in vivo (36). Radiation response in vitro may be influenced by a variety of factors including oxygen effects, the presence of an immune system, antioxidant levels, DNA repair and the extracellular environment that are not adequately modeled in vitro. As an example, the in vitro clonogenic survival assays with ketones were conducted in 21% oxygen which is significantly higher than the partial pressure of oxygen in solid tumors of animals fed ketogenic diets (37). Furthermore, therapeutic radiation doses also initiate a cascade of signaling events involving cytokines and oxidant-producing pathways that further induce activation of the immune system and inflammation that contributes to tissue damage and radiation responses that is not adequately modeled in vitro (38). Our data suggest that when studying the effects of ketogenic diets on radiation responses that in vivo model systems may be more appropriate than in vitro model systems.

The current study supports the conclusion that ketogenic diets are well tolerated by tumor-bearing animals receiving concurrent radio-chemo-therapy. These results also support the conclusion that feeding a ketogenic diet could serve as an easily implemented adjuvant for improving responses to radio-chemo-therapies in the treatment of lung cancer. Finally, these data also support the hypothesis that a ketogenic diet enhances radiation responses in lung cancer xenografts by a mechanism involving oxidative stress and inhibition of cancer cell proliferation in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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