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Obesity Promotes Aerobic Glycolysis in Prostate Cancer Cells

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Abstract

Obesity is the leading preventable comorbidity associated with increased prostate cancer-related recurrence and mortality. Epidemiological and clinical studies indicate that a BMI >30 is associated with increased oxidative DNA damage within the prostate gland and increased prostate cancer-related mortality. Here we provide evidence that obesity promotes worse clinical outcome through induction of metabolic abnormalities known to promote genotoxic stress. We have previously reported that blood serum derived from obese mice may enhance the proliferative and invasive potential of human prostate cancer cell lines *ex vivo*. Here we show that a 1 hour exposure of LNCaP or PacMetUT1 prostate cancer cell lines and non-malignant RWPE-1 prostate epithelial cells to 2% serum from obese mice induces markers of aerobic glycolysis relative to those exposed to serum from non-obese mice. This metabolic change was correlated with accumulation of reactive oxygen species (ROS) and increased frequency of DNA double-strand breaks. Interestingly, *N-tert*-Butylhydroxylamine (NtBHA), an antioxidant, significantly suppressed markers of aerobic glycolysis in the cells exposed to Ob serum, suggesting that ROS contributes to a metabolic shift toward aerobic glycolysis. Here we describe obesity-induced changes in key metabolic markers that impact prostate cancer cell progression and explore the role of antioxidants in ameliorating these effects.

Keywords

experimental; prostate cancer and metabolism

Introduction

Obesity is a global epidemic strongly implicated in promoting the progressive etiology of cardiovascular disease [1–3], type II diabetes [4, 5], and certain forms of cancer, including prostate carcinoma [6, 7]. Although obesity is not significantly associated with low grade prostate cancer, increasing evidence suggests that high adiposity, BMI and waist circumference are positively correlated with higher grade Gleason scores [6], higher rates of biochemical recurrence [3, 8] and a 50% increase in prostate cancer mortality [7, 9]. Despite accumulating evidence indicating that obesity is a direct and modifiable risk factor

impacting prostate cancer progression, the affected biological pathways are relatively unexplored. Numerous pre-clinical and clinical studies have demonstrated differences in the metabolic profiles of lean versus obese subjects, including significant differences in the concentration of growth factors [10, 11], cytokines [11, 12] and prostaglandins [11, 13]. This obesity-induced modulation of the growth factor signaling milieu in blood serum may enable an earlier energetic switch toward glycolysis, also known as the Warburg effect, thereby promoting prostate cancer progression. The Warburg effect, whereby cancer cells partially forego mitochondrial oxidative phosphorylation (OXPHOS) in preference of aerobic glycolysis, is posited to provide a growth advantage to rapidly dividing cells by facilitating conversion of nutrient uptake into biomass [14]. Despite the inherent inefficiency of glycolysis in generating sufficient ATP to meet the energetic demands of rapidly proliferating cells, it has been suggested that high rates of aerobic glycolysis may simultaneously satisfy the cellular demand for ATP as well as provide the biochemical intermediates necessary to generate the lipid, protein and DNA precursors needed for sustained rapid growth and proliferation [15].

Consistent with these observations, we provide *ex vivo* evidence that serum from obese mice enhances aerobic glycolysis in malignant and non-malignant human prostate cells through accumulation of cellular reactive oxygen species (ROS), which stabilize HIF-1 α expression and activity [16]. HIF-1 α , a transcriptional regulator of genes involved in aerobic glycolysis, mediates glucose reprogramming through induction of glucose transporter-1 (GLUT1), lactate dehydrogenase (LDHA) and pyruvate kinase (PK) [17]. PKM2, the major isoform of PK expressed in rapidly proliferating human fibroblasts, stem cells and a variety of solid tumors, is the final enzyme in glycolysis [18]. Upregulation of PKM2 in cancer cells rapidly accelerates glycolysis, production of ATP and conversion of nutrients into biomass [15], thus facilitating cellular proliferation.

In the present study, we show that circulating factors in the blood serum of obese mice (Ob serum) induce markers of aerobic glycolysis and ROS relative to serum derived from non-obese (non-Ob serum) mice. To obtain blood serum from the respective weight classes, mice were maintained on either a diet containing 60% calories from fat versus isonutrient control diet containing 10% calories from fat. LNCaP, PacMetUT1 and the spontaneously telomerized RWPE-1 cell lines cultured in Ob serum or non-Ob serum displayed an accumulation of ROS, as indicated by the intracellular conversion of a nonfluorescent 2',7'-dichlorfluorescein – diacetate (DCFH-DA) to a fluorescent 2',7' – dichlorfluorescein (DCF) probe. To validate the modulation of ROS in response to Ob serum, we assessed the frequency of γ -H2AX foci, a robust indicator of ROS-induced DNA Double-Strand Breaks (DSBs). We observed a massive induction of γ -H2AX in cells grown in Ob serum at levels consistent with those observed after exposure to physiologic pulses of hydrogen peroxide, confirming that Ob serum results in increased oxidative stress. Further, the increase in ROS was associated with stabilization of HIF-1 α and increased PK activity. Conversely, expression of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA as substrate for mitochondrial OXPHOS, was diminished after exposure to Ob serum. Taken together, the increase in PK activity and decrease in PDH expression after exposure to Ob serum suggests that circulating factors within the Ob serum can shift the metabolism of

cells, *ex vivo*, toward aerobic glycolysis. To further demonstrate the link between increased ROS and PK activity, cells were pretreated with *N-tert*-Butylhydroxylamine (NtBHA), a known mitochondrial antioxidant. NtBHA reversed the obesity-induced formation of γ -H2AX foci and suppressed PK activity, suggesting a role of antioxidants in mitigating aerobic glycolysis. In conclusion, these data suggest that obesity may accelerate prostate cancer progression through the induction of ROS-induced aerobic glycolysis.

Materials & Methods

Cell Lines

The LNCaP cell line was purchased from the American Tissue Type Culture Collection (ATTC) and grown in RPMI supplemented with 10% fetal bovine serum (FBS) in a 5% (v/v) CO₂ humidified incubator at 37° C. As previously described, the PacMetUT1 cell line was derived from a 57-year-old male with known prostate cancer and grown in RPMI + 10% FBS under standard tissue culture conditions. The HPV-18-transfected, non-malignant RWPE-1 prostate epithelial cell line was purchased from the ATTC and was grown in RPMI + 10% FBS under standard tissue culture conditions.

Animals

Ten male animals on a C57BL/J background were used for these experiments. The animals were bred and maintained at the AAA-LAC accredited Animal Resources Center (ARC) facility at the University of Texas at Austin. After weaning, at between 3 to 4 weeks of age, animals were randomly sorted onto either a diet-induced obesity (DIO) diet consisting of 60% of calories from fat (Research Diets, D12492), or an isonutrient control (Cntrl.) diet (Research Diets, D12450) containing 10% of calories from fat. Dietary intake and weights were observed for 12 weeks and then animals euthanised with CO₂ for collection of whole blood by cardiac heart puncture. Blood serum was separated after 1 hr by centrifugation at 2,000 RPM and serum aliquoted and frozen at -80° C for subsequent *ex vivo* studies.

Western Blot Analysis

LNCaP, PacMetUT1 and RWPE-1 cells were seeded and cultured under standard tissue culture conditions for 48 hrs, then rinsed in 1 × PBS and incubated overnight in serum-free media with or without antioxidants. The next morning, 2% FBS, 2% Ob serum or 2% non-Ob serum (vol./vol.) was added directly to respective plates of cells and incubated for 1 hr then rinsed twice with 1 × PBS and cells harvested for preparation of lysates in 1 × lysis buffer (50mM Tris-HCL, pH 7.5, 120 mM NaCl, 1% NP-40, 1 mM EDTA pH 8.0, 50 mM NaF, 40 mM b-glycerolphosphate, 100 mM Na orthovanadate, 1 mM benzamidine and 1 mM PMSF). Protein lysates were subjected to immunodetection with rabbit anti-HIF-1 alpha (Cell Signaling); mouse anti- γ H2AX (Upstate); or rabbit anti-PDH (Cell Signaling). HPRT-luminescent signal was acquired using a Syngene gel documentation system and signal was standardized to actin for densitometric analysis and conditions normalized to serum-free media for relative comparison in three independent experiments.

Reactive Oxygen Species Assay

Intracellular levels of reactive oxygen species (ROS) were assessed using a cell-permeable fluorogenic 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) probe (Cell Biolabs) as previously described. Briefly, LNCaP and PacMetUT1 prostate cancer cells were cultured for flow-cytometric assessment on 6-well dishes or for microscopy directly on an 8-chamber glass Lab-Tek™ II chamber slide system. Cells were cultured for 48 hrs, then rinsed twice in 1 × PBS and incubated overnight in serum-free media. The next morning, either 2% fetal bovine serum, 2% Ob mouse serum or 2% non-Ob mouse serum was added directly to the cells for 1 hr and then rinsed with 1 × PBS. The 20× DCFH-DA was diluted and the cells incubated for 1 hr, then rinsed 3× in PBS and fixed for microscopy as follows:

For microscopy, LNCaP and PacMetUT1 cells were grown on slides and exposed to serum from obese or control weight mice in the presence of the ROS probe, DCFH-DA, and fixed in 4% paraformaldehyde/PBS solution at room temperature for 5 min. Cells were counterstained with 200 ng/ml DAPI for detection of nuclei and fluorescent images of DAPI and DCFH-DA stained cells were captured on a Leica DM 4000B epifluorescent microscope at identical exposure conditions in three independent experiments to ensure reproducibility.

For flow cytometry, serum exposed LNCaP cells were incubated in 1× DCFH-DA, rinsed 3× in PBS and harvested for flow cytometry as follows: Adherent cells growing in 6-well plates were trypsinized and harvested after exposure to respective conditions. Cells were washed with PBS and resuspended in PBS containing 0.1 mg/ml RNase and 5 µg/ml PI. Live cells were then run on an Accuri C6 Flow Cytometer and final data analysis performed using FlowJo software. 3×10^4 events were recorded for each sample in three independent experiments.

Immunofluorescence

LNCaP and PacMetUT1 cell lines were grown and treated directly on an 8-chamber glass Lab-Tek™ II chamber slide system as described above. After exposure to the various 2% serum conditions for 1 hr, slides were fixed and permeabilized in a 0.1% Triton X-100/PBS solution at RT for 5 min for subsequent immunodetection of double-strand DNA breaks using mouse anti-γH2AX (Bethyl Laboratories). Expression of HIF-1α, a mediator of aerobic glycolysis, was determined using mouse anti-HIF-1α (Calbiochem) in three independent experiments. FITC or Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) allowed detection of primary antibody on a Leica DM 4000B epifluorescence microscope.

Pyruvate Kinase Activity

Rates of glycolysis were determined in LNCaP and PacMetUT1 cells. Briefly, cells were seeded on 6-well dishes and cultured for 48 hrs. After 48 hrs, cells were rinsed in 1 × PBS and incubated overnight in serum-free media. The next day, 2% fetal bovine serum, 2% obese mouse serum or 2% control weight mouse serum was directly added to the cells for 1 hr and the cells harvested for determination of pyruvate kinase activity (BioVision). Activity was measured in three independent experiments at OD 570 nm according to the formula: PK Activity = $B/(T2-T1) \times V \times \text{Sample dilution factor} = \text{nmol/min/ml} = \text{mU/ml}$.

Statistical analysis of data

Exposure of cells to respective serum conditions or SFM was conducted using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diego, CA). Results were compared using a one way ANOVA with a Tukey's Posthoc test. Results were considered statistically significant at $P = 0.05$.

Results

Effect of DIO-diet on mouse weights

To generate Ob and non-Ob mouse serum for subsequent *ex vivo* studies, C57BL/6 mice were weaned at 21 days, then randomized and maintained on DIO or Cntrl diet for 12 weeks. Ob mice achieved an average weight of 43 grams versus non-Ob mice, which averaged 27 grams (Fig. 1). After collection of whole blood by cardiac puncture, internal examination revealed elevated subcutaneous and abdominal adiposity with excess accumulation in the liver and digestive system, indicating altered pathology (*data not shown*).

Exposure to Ob serum induces oxidative stress in prostate cancer cells

To determine the effect of circulating factors in the blood serum of Ob mice in promoting oxidative stress, we initially exposed LNCaP cells to serum from Ob or non-Ob mice. Flow cytometry was used to assess relative ROS accumulation in the cells after a 1 hr exposure to 2% Ob, non-Ob or fetal bovine serum and serum free media using the DCFH-DA probe. Using the DCFH-DA probe which becomes fluorogenic when oxidized by a variety of ROS, we measured the relative oxidative stress levels of these cells when exposed to the respective sera. By flow cytometry, we observed a 10-fold accumulation of ROS in LNCaP cells grown in 2% Ob serum versus serum-free media, and a 2-fold accumulation compared to cells grown in 2% non-Ob serum (Fig. 2A). To visually confirm the flow analysis, both LNCaP and PacMetUT1 prostate cancer cell lines were exposed to 2% respective serum or serum-free media for 1 hr and fluorescent images captured to compare relative intensities of DCFH-DA probe among the treatments. PacMetUT1 cells displayed a dramatic increase in ROS, nearly to the level observed in the 62.5 μ M hydrogen peroxide control treatment. Both the LNCaP and PacMetUT1 cell lines displayed elevated ROS after exposure to Ob serum (Fig. 2 B).

A consequence of increased cellular ROS includes induction of genomic DSBs. Relative levels of DSBs were determined by immunofluorescence for the phosphorylated histone variant (γ -H2AX) which translocate to sites of DSBs. LNCaP and PacMetUT1 cells grown in 2% FBS or 2% non-Ob serum exhibited low basal levels of γ -H2AX foci, while cells exposed to 2% Ob serum exhibited robust nuclear γ -H2AX foci (Fig. 2C). Few DSBs were detected in the non-Ob serum exposed cells, which is consistent with the absence of appreciable levels of ROS in either cell line.

Effects of Ob serum on genotoxic stress and metabolism

To confirm that DSBs are formed after exposure to 2% Ob serum, we quantified the expression of γ -H2AX via Western blot analysis in LNCaP, PacMetUT1 and RWPE-1 cells.

Here we observe that Ob serum strongly induces γ -H2AX foci in both the malignant and non-malignant cells, suggesting that circulating factors within the serum are capable of modulating oxidative stress to promote formation of DSBs. As DNA damage is a means of acquiring genetic mutations that drives tumorigenicity, our data is consistent with a model in which obesity promotes a more aggressive prostate cancer phenotype through induction of DSBs. HIF-1 α , a key transcriptional activator of enzymes involved in glycolysis, was also strongly induced by Ob serum compared to non-Ob in the malignant and non-malignant cells. Interestingly, the most dramatic differences in HIF-1 α and γ -H2AX expression are observed in the RWPE-1 cells, suggesting that factors found within the obese serum affect non-malignant cells as well as malignant (Fig. 3A). Next, we confirmed HIF-1 α induction and nuclear translocation in response to Ob-serum by immunofluorescence. In agreement with the immunoblots, HIF-1 α levels increased after exposure to 2% Ob serum compared to 2% FBS or non-Ob serum. Further, the pattern of HIF-1 α staining indicates strong nuclear localization in 2% Ob serum, while the cells cultured in 2% non-Ob serum exhibited lower relative levels, nuclear exclusion and accumulation at the cell periphery (Fig. 3B).

Antioxidants suppress the effects of Ob serum on genotoxic stress and metabolism

To demonstrate that circulating factors within Ob serum directly affect HIF-1 α stabilization as well as the formation of γ -H2AX foci in LNCaPs, cells were pretreated for 24 hrs in NtBHA, a potent mitochondrial-targeted antioxidant. After 1 hr exposure to Ob serum, HIF-1 α stability was strongly induced relative to non-Ob, as previously observed (Fig. 3). Further, overnight pretreatment with NtBHA effectively diminished Ob serum-induced HIF-1 α levels by half. No additional decrease of HIF-1 α was observed among the non-Ob and non-Ob + NtBHA conditions. Similarly, NtBHA significantly suppressed the Ob serum-induced γ -H2AX, with no additional effects seen in the non-Ob condition (Fig. 4A). PDH, the rate-limiting step in pyruvate processing to acetyl-CoA during OXPHOS, was also observed to be suppressed by Ob serum (Fig. 4B), although no statistically significant differences were observed after combining three independent experiments (densitometry), the trend toward decreased PDH expression was consistently seen across all replicates. This is consistent with the Warburg effect, in which cells forgo OXPHOS in preference of aerobic glycolysis. Our results indicate that Ob serum, to a greater extent than non-Ob serum, promotes the Warburg effect through suppression of PDH. Intriguingly, overnight pretreatment with NtBHA restored PDH expression among both the Ob and non-Ob treatments, suggesting that antioxidants may effectively restore mitochondrial pyruvate processing.

Ob serum modulates PK activity

PK is the final enzyme involved in glycolysis which catalyzes the transfer of inorganic phosphate from phosphoenolpyruvate to ADP, yielding ATP and pyruvate. Using a fluorescence-based PK activity assay, we assessed the effect of exposure to Ob serum on rates of aerobic glycolysis. We observe significant increases in PK activity in LNCaP cells after exposure to Ob serum (3.1 mU/ml versus non-Ob 2.7 mU/ml). Both serum conditions were elevated compared to 2% FBS 2.5 mU/ml, or SFM 2.2 mU/ml.

Discussion

Obesity is a known risk factor for progression to advanced prostate cancer. Because obesity is known to deregulate metabolism, a new hallmark of cancer [19, 20] and since metabolic disorders are frequently accompanied by systemic inflammation and oxidative stress [21, 22], we assessed its impact on prostate cancer cell metabolism and DNA damage as indicators of tumorigenicity. Here we show that human prostate cancer cell lines exposed to Ob serum from mice maintained on a 60% calories from fat DIO-diet (Fig. 1), display a rapid and pronounced accumulation of ROS (Fig. 2A & B) which is accompanied by formation of γ -H2AX foci, an indicator of DSBs (Fig. 2C). Since DSBs are highly cytotoxic lesions that result in mutations or chromosome translocations when inaccurately repaired by nonhomologous end joining or homologous (VDJ) recombination, these findings provide compelling mechanistic evidence for the impact of obesity on cancer progression. In conjunction with these genetic events, we also observe evidence that acute exposure to Ob serum alters the metabolic profile of prostate cancer cells in favor of aerobic glycolysis. Non-malignant RWPE-1 prostate epithelial cells and malignant LNCaP and PacMetUT1 cells all exhibit strong induction of HIF-1 α after exposure to Ob serum compared to non-Ob. HIF-1 α is a ROS-inducible transcription factor which is normally hydroxylated and marked for ubiquitin-mediated proteosomal degradation [23]. Under conditions of hypoxia or increased ROS, HIF-1 α accumulates and initiates transcription of key enzymes involved in glycolysis [24–27]. Interestingly, HIF-1 α accumulates in both malignant and non-malignant cells exposed to Ob serum (Fig. 3A). Because accumulation of HIF-1 α does not directly demonstrate transcriptional activity, we next determined the subcellular localization by IF (Fig. 3B). Strong nuclear HIF-1 α staining was observed in the LNCaP and PacMetUT1 cells exposed to Ob serum relative to non-Ob. Further, HIF-1 α staining in the non-Ob serum exposed cells seemed concentrated along the periphery of the cell membrane, thereby precluding its transcriptional activity. Since HIF-1 α is known to modulate glycolysis through transcriptional regulation of PK, which catalyzes the substrate level phosphorylation of ADP to ATP, we utilized a fluorescence-based PK activity assay to assess the effect of Ob serum on the rates of aerobic glycolysis (Fig. 5). Here we observe a statistically significant increase in PK activity when comparing the Ob and non-Ob serum conditions. After 10 minutes in Ob or non-Ob serum, PK activity diverges out to the completion of the assay at 20 minutes. This rapid and continuous increase in PK activity after exposure to Ob serum suggests an acute and lasting effect. The implications of this data are even more compelling when combined with complimentary data in our lab which have demonstrated that LNCaP cells exposed to Ob serum for 1 hour and subsequently injected into athymic nude mice undergo a doubling in tumor volume after 12 weeks (*data not shown*). Together, these data suggest a model in which circulating factors in the serum of obese mice promote an acute and sustained increase in tumorigenicity through deregulated metabolism.

Although the efficacy of dietary antioxidants in cancer therapy have shown mixed clinical results [28–30], few studies have focused on their role in reversing the contributions of obesity on prostate cancer. Our *in situ* studies demonstrate that NtBHA suppresses Ob serum-induced HIF-1 α as well as formation of γ -H2AX foci (Fig. 4A), affirming that ROS is the underlying cause of HIF-1 α and γ -H2AX induction. To determine if the observed

increase HIF-1 α affected downstream metabolic pathways, such as OXPHOS, we performed an immunoblot for pyruvate dehydrogenase (PDH) expression in LNCaP cells pretreated +/- NtBHA overnight and then exposed to Ob serum 1 hour. We observed that exposure to Ob serum reduced PDH expression and that NtBHA restored PDH even in the presence of Ob serum. Because PDH is the rate-limiting factor in the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) during OXPHOS, suppression of PDH by Ob serum would inhibit OXPHOS and provide more substrate for aerobic glycolysis. That this effect was reversed by NtBHA suggests that antioxidants may reverse obesity-induced metabolic reprogramming (Fig. 4B). While it is known that the majority of intracellular ROS are produced within peroxisomes and mitochondria, future studies are required to precisely determine the mechanism of Ob-induced ROS. Our ongoing studies indicate that Ob serum modulates the balance of mitochondrial biogenesis and mitophagy, resulting in the aggregation of expended mitochondria that generate and leak ROS.

Taken together, we propose a model in which obesity precipitates two concurrent events that confer a selective growth advantage in prostate cancer cells: First, obesity drives aerobic glycolysis, enabling rapid and sustained proliferation. Second, obesity induces genotoxic stress to facilitate the acquisition of protumorigenic mutations. Together, the rapid rate proliferation coupled with increased rates of mutations present a state of positive selective pressure that drives prostate tumorigenicity. Although previous studies have described the role of oxidative stress in activating HIF-1 α to modulate glycolysis [31], we are the first to describe a potential model in which obesity promotes metabolic reprogramming in prostate cancer cells. Moreover, we have identified that antioxidants, such as NtBHA may be developed as potential therapeutic agents to mitigate the effects of obesity on prostate cancer progression.

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Figure 1. Mouse weights on DIO and Isonutrient Control Diets

Three week old mice were weaned then randomized and maintained on 60% calorie from fat DIO-diet or 10% calorie from fat control diet for 12 weeks.

Figure 2. Induction of ROS by DIO-mouse serum in LNCaP and PacMetUT1 prostate cancer cell lines

Flow cytometry; * $p=0.002$ (A) and fluorescence microscopy (B) were used to assess relative levels of ROS (green) after growth in DIO or control serum conditions using the DCFH-DA probe. Immunofluorescent staining of the histone variant γ -H2AX (C) was used as an indicator of DNA Double-Strand Breaks (green). Cells were counterstained with DAPI for visualization of cellular nuclei (blue).

Figure 3. Ob serum modulates HIF-1 α and γ -H2AX in malignant and non-malignant prostate cells

Ob serum induces γ -H2AX and HIF-1 α expression in the LNCaP and PacMetUT1 prostate cancer cell lines as well as the non-malignant, spontaneously transformed RWPE-1 cell line. Immunoblot detection of γ -H2AX and HIF-1 α expression levels (A). Indirect Immunofluorescence showing Hif-1 α levels and subcellular localization in malignant cells (B).

Figure 4. Antioxidants attenuate obesity-induced expression of HIF-1 α and γ -H2AX
Immunoblot analysis of LNCaP cells shows the effect of the antioxidant, NtBHA on Ob serum-induced HIF-1 α and γ -H2AX expression (A) or expression of PDH (B).

Figure 5. Effect of Ob-serum on PK activity

Relative rates of pyruvate kinase activity after exposure to Ob or non-Ob serum.