Metformin Reduces Prostate Tumor Growth, in a Diet-Dependent Manner, by Modulating Multiple Signaling Pathways

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Abstract

Prostate-cancer is strongly influenced by obesity, wherein metformin could represent a promising treatment; however, the endocrine metabolic/cellular/molecular mechanisms underlying these associations and effects are still unclear. To determine the beneficial antitumoral effects of metformin on prostate cancer progression/aggressiveness and the relative contribution of high-fat diet (HFD; independently of obesity), we used HFD-fed immunosuppressed mice inoculated with PC3 cells (which exhibited partial resistance to diet-induced obesity) compared with low-fat diet (LFD)-fed control mice. Moreover, gene expression analysis was performed on cancer-associated genes in the xenografted tumors, and the antitumorigenic role of metformin on tumor (PC3/22Rv1/LNCaP) and normal (RWPE1) prostate cells was evaluated. The results demonstrate that HFD is associated with enhanced prostate cancer growth irrespective of body weight gain and endocrine metabolic dysregulations and that metformin can reduce prostate cancer growth under LFD but more prominently under HFD, acting through the modulation of several tumoral-associated processes (e.g., cell cycle, apoptosis, and/or necrosis). Moreover, the actions observed in vivo could be mediated by the modulation of the local expression of GH/IGF1 axis components. Finally, it was demonstrated that metformin had disparate effects on proliferation, migration, and prostate-specific antigen secretion from different cell lines. Altogether, these data reveal that metformin inhibits prostate cancer growth under LFD and, specially, under HFD conditions through multiple metabolic/tumoral signaling pathways.

Implications: The current study linking dietary influence on metformin-regulated signaling pathways and antitumoral response provides new and critical insight on environment-host interactions in cancer and therapy.

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Introduction

Prostate cancer is the second most common type of cancer among men in developed regions, and the age at diagnosis is decreasing (1). Obesity, a multifactorial disease with increasing incidence in developed countries and strongly linked to high-fat diet (HFD) consumption, is associated with development/progression of several cancer types (2). This association is of special relevance in prostate cancer, an endocrine-related cancer strongly influenced by the androgenic status (3). Of note, obesity seems to influence prostate cancer development as much as obesity is related with 15% to 20% of cancer incidence and normal (RWPE1) prostate cells was evaluated. The results demonstrate that HFD is associated with enhanced prostate cancer growth irrespective of body weight gain and endocrine metabolic dysregulations and that metformin can reduce prostate cancer growth under LFD but more prominently under HFD, acting through the modulation of several tumoral-associated processes (e.g., cell cycle, apoptosis, and/or necrosis). Moreover, the actions observed in vivo could be mediated by the modulation of the local expression of GH/IGF1 axis components. Finally, it was demonstrated that metformin had disparate effects on proliferation, migration, and prostate-specific antigen secretion from different cell lines. Altogether, these data reveal that metformin inhibits prostate cancer growth under LFD and, specially, under HFD conditions through multiple metabolic/tumoral signaling pathways.

Implications: The current study linking dietary influence on metformin-regulated signaling pathways and antitumoral response provides new and critical insight on environment-host interactions in cancer and therapy.

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cancer growth in in vivo xenograft mouse models under HFD conditions (15). However, to the best of our knowledge, it has not been proven to date whether the antitumoral actions of metformin in vivo are related to the metformin-mediated improvement of the metabolic condition and/or to a direct action on tumoral prostate cells.

Therefore, a growing body of independent evidence supports the association between endocrine metabolic alterations (such as obesity) and the development and progression of prostate cancer, as well as the promising role of metformin in controlling prostate cancer outcome; however, to the best of our knowledge, to date, no studies have specifically focused on simultaneously: (i) determining whether HFD consumption, independently of obesity and its associated endocrine metabolic alterations, might be associated with the progression/aggressiveness of prostate cancer in vivo, compared with a low-fat diet (LFD) consumption; (ii) determining whether the antitumoral effects of metformin in vivo are similar under LFD and HFD conditions and, most importantly; (iii) identifying the precise cellular/molecular mechanisms underlying these potential in vivo associations/effects under HFD/LFD conditions.

To achieve these goals, we used a combination of different in vivo and in vitro approaches, including the use of a mouse model with partial resistance of HFD-induced obesity (16–18) to dissect out the role of HFD-feeding or LFD-feeding intake and metformin treatment in the development and progression of xenografted prostate cancer cells without the confounding effects of dysregulated endocrine metabolic conditions as well as, normal-like and/or prostate cancer cell lines to evaluate the in vivo effect of metformin in proliferation, migration, and/or prostate-specific antigen (PSA) secretion.

Materials and Methods

Animal model

All experimental procedures were carried out following the European Regulations for Animal Care, in accordance with guidelines and regulations, and under the approval of the University/Regional’s Government Research Ethics Committees. Seven-week-old male immunodeficient NUDE Foxn1nu/C0Foxn1nu (n = 22; Janvier Labs) were housed in sterile filter-capped cages and maintained under standard conditions of temperature (22°C–24°C), with free access to sterilized diet and water. Mice were fed with an LFD (Research Diets; D124508; 10% Kcal fat, 70% Kcal carbohydrates, 20% Kcal proteins; 3.85 Kcal/g) or HFD (Research Diets; D12492; 60% Kcal fat, 20% Kcal carbohydrates, 20% Kcal proteins; 5.24 Kcal/g) for 12 weeks (Supplementary Table S1), starting at 6 weeks of age (micronutrients were equivalent in both diets). Two weeks after starting the diet, 2.5 × 10⁶ human prostate cancer cells (PC3) were resuspended in 100 μL BME matrix (Cultrex Basement Membrane Matrix; Trevigen) and subcutaneously xenografted into the right and left back flanks of each mouse. To estimate food and calorie intake, singled house mice were provided with preweighted food and, 5 days later, remaining food was collected and weighted. Three weeks after the inoculations, metformin (Alfa Aesar, Thermo Fisher) or vehicle treatments were started and maintained during 7 weeks (250 mg/Kg/day in drinking water, according to the mouse water intake; dose of metformin based in previous studies; refs. 19, 20). Mice were randomly assigned to each diet and treatment, comprising 4 experimental groups: groups 1 and 2 fed a LFD without metformin (LFD-H2O, n = 5) and with metformin (LFD-Met, n = 6), respectively, and, groups 3 and 4 fed an HFD without metformin (HFD-H2O, n = 5) and with metformin (HFD-Met, n = 6), respectively (Fig. 1A).

Body weights and tumor volumes were measured once a week with a digital caliper till the day of euthanasia. Tumor volume was calculated as previously reported (21). After 7 weeks of treatment with and without metformin, mice were killed by decapitation without anesthesia. The whole procedure and the experiment to generate this mouse model was performed normally, and no mouse died during the experiment. Trunk blood was collected and tumors and prostate glands were excised and weighed. A portion of the tumor was snap-frozen in liquid nitrogen for protein and RNA expression analyses, and the remaining tissue was fixed in 10% formalin to obtain sections for histologic analysis. Body composition was also evaluated using the Body Composition Analyzer E26-240-RMT (EchoMRI LLC) just before the killing.

Assessment of plasma hormones

Trunk blood was immediately mixed with MiniProtease inhibitor (Roche), placed on ice, centrifuged, and plasma was stored at −80°C. Commercial ELISA kits were used to measure circulating levels of insulin, leptin, GH (EZRM-13K, EZM-82K, EZRMGH-45K, respectively; Millipore), corticosterone, IGFI (AC-14F1, AC-18F1, respectively, Immunodiagnostic Systems Kij, and total PSA (RAB0331, Sigma-Aldrich) following the manufacturer’s instructions. All the information regarding specificity, detectability, and reproducibility for each of the assays can be accessed at the web site of the company.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA from tumors and whole normal prostates was isolated using the AllPrep DNA/RNA/Protein Mini-Kit following manufacturer’s instructions, treated with DNase (Qiagen), and quantified with the NanoDrop2000 spectrophotometer (Thermo Fisher). RNA (1 μg) was reverse-transcribed using random hexamer primers (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher). qPCR reactions were performed using the Brilliant III SYBR-Green QPCR Master Mix (Stratagene) in the Stratagene Mx3000p system as previously reported (22). To control for variations in the amount of RNA used and the RT reaction efficiency, mRNA copy numbers were adjusted by a normalization factor obtained from the expression of β-ACTIN and HPRT for tumor samples and β-Actin, Hprt, and cyclophilin for mouse prostates (housekeeping genes whose expression did not vary between experimental groups) using the Genorm application. Standard curves were run in parallel to quantify the copy number in each sample. Specific primers (Supplementary Table S2) were designed with Primer3 software and validated as reported previously (23).

Western blotting

The protein extracted from the tumor with the AllPrep DNA/RNA/Protein Mini-Kit (previously referred) was resuspended in SDS-DTT buffer, sonicated, heated for 5 minutes at 95°C, separated on 10% acrylamide gels, and electrophoretically transferred to Hybond-ECL nitrocellulose membranes...
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(TBS-T), and incubated overnight (4°C) with primary antibodies 1:1,000 against phospho-Ser473 Akt (#9271S; Cell Signaling Technology), total-AKT (#9272; Cell Signaling Technology), AKT3 (ab152157, Abcam), GADD45G (ab196774, Abcam), IL12A (ab131039, Abcam), or 1:500 for TNFRe (sc-52746, Santa Cruz Biotechnology), cyclin D2 (ab81359, Abcam) in TBS-T, 5% nonfat dry milk. Then, blots were incubated with HRP-conjugated goat-anti rabbit IgG (#7076, Cell Signaling Technology) in 5% dry milk, TBS-T for 1 hour, washed, and exposed (5 minutes) to Clarity Western-ECL Blotting-Substrate (1705060; Bio-Rad Laboratories). Films were scanned using ImageQuant Las 4000 system (GE Healthcare Europe GmbH) and images were analyzed with ImageJ.

Prostate cell lines
Three widely accepted human prostate cancer-derived cell lines, PC3 cells (ATCC CRL-1435), LNCaP (ATCC CRL1740), 22Rv1 (ATCC CRL2505), and one normal prostate cell line RWPE1 (ATCC CRL-11609) were cultured and maintained following manufacturer's instructions. The cell lines were validated by the analysis of short tandem repeat (STR; GenePrint 10 System, Promega) and checked for mycoplasma as previously reported.[22] All cell lines were maintained at 37°C and 5% CO₂, under sterile conditions.

Measurements of proliferation
To evaluate the proliferative response of the normal and tumoral prostate cells to metformin, a dose–response experiment (1, 2.5, and 5 mmol/L) was performed in RWPE-1, PC3, LNCaP, and 22Rv1 cells. Specifically, cells were seeded in 96-well plates at a density of 5,000 per well (4 wells/treatment in at least 3 independent experiments) and treated with metformin in medium (specific for each of the cell lines used supplemented with 5% FBS; Sigma). Cell proliferation was measured in a Flex Station 3 (Molecular Devices) at 24, 48, and 72 hours of incubation using Alamar-Blue assay (Life Technologies), as previously described.[22] Medium was replaced by fresh medium (with the different treatments) immediately after each measurement.

Measurements of migration capacity
The ability of PC3 and RWPE1 cells to migrate in response to metformin was evaluated by wound-healing assay as previously reported.[22] Briefly, cells were cultured at subconfluence in 12-well plates and serum-starved for 24 hours. The wound was made using sterile pipette tips, wells were rinsed in PBS, and incubated for 14 hours with medium without FBS. Pictures were taken using a Motic AE2000 camera (Motic Europe). Migration was evaluated as the area recovered 14 hours after the wound versus the area just after the wound was performed using the ImageJ software (RSB). At least n = 3 experiments were performed in independent days, in which 3 random pictures along the wound were acquired per well.

Histologic analysis
Right after the tumor excision, a section of each tumor obtained from the mice included in the 4 experimental groups generated (in vivo experimental model described above: LFD-H₂O, LFD-Met, HFD-H₂O, and HFD-Met; n = 5–6) was fixed in 10% formalin, paraffin-embedded, and sectioned in 7-μm sections for hematoxylin and eosin staining by the Laboratory of Histology at UCAIB (IMIBIC). All sections obtained in each experimental group were examined for tumor necrosis extension and mitotic activity by 2 expert anatomopathologists (in a blinded fashion) using an optic NIKON eclipse 50i microscope.

Array
nCounter PanCancer Pathways Panel kit (GXA-PATH1-12, NanoString Technologies) was used and performed at the Laboratory of Genetics at UCAIB (IMIBIC) to simultaneously examine the expression of 730 genes associated with cancer (i.e., 606 genes representing all major cancer pathways and 124 key cancer driver genes). Briefly, after analyzing the quality of all samples using microelectrophoresis, 100 ng of RNA from 3 independent tumoral samples (samples with the best quality) from the 4 experimental groups generated (in vivo experimental model: LFD-H₂O, LFD-Met, HFD-H₂O, and HFD-Met) were loaded in the plate provided in the nCounter PanCancer Pathways Panel Kit, and the experiment was run following manufacturer's protocol. The data were analyzed using the nSolverAnalysisSoftware3.0.22 from NanoString Technologies with the PanCancer Pathways Analysis Module using 40 genes as housekeeping genes. All specific target sequences and panel details are available on the manufactures webpage.

Statistical analysis
Samples from all groups were processed at the same time. All values are expressed as mean ± SEM or compared with the corresponding controls (set at 100%). In all cases, a Kolmogorov–Smirnov test was applied to explore the normality of the values and, subsequently, parametric or nonparametric tests were implemented to analyze the statistical differences. Particularly, when only 2 groups were compared, the t test was used. However, multiple comparisons were performed using a 2-way ANOVA followed by post-hoc analysis (Fisher). P < 0.05 was considered statistically significant. When P values ranged between <0.1 and >0.05, a trend for significance was indicated where appropriate. All statistics analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software Inc.).

Results
Influence of diet on body weight, body composition, metabolic status, food intake, and normal prostates
The 4 experimental groups of immunodeficient mice were fed an LFD or an HFD at 7 weeks of age, subcutaneously injected with PC3 cells at 9 weeks of age, or not, with metformin at 12 weeks of age for 7 additional weeks (LFD-H₂O, LFD-Met, HFD-H₂O, and HFD-Met) displayed similar body weight gaining throughout the study (Fig. 1B) and, consequently, presented similar body weight at the end of the study. In addition, fat and lean mass percentages (Fig. 1C) and fed glucose and insulin levels at the moment of euthanasia (Fig. 1D) were similar between groups. Interestingly, although corticosterone levels were not altered by diet or metformin treatment, leptin levels were significantly increased by diet (P < 0.01 by two-way ANOVA), being
these differences especially drastic in the control (H2O)-treated group (Fig. 1D). Supporting the body weight results, mice under an HFD ingested less food than LFD mice, whereas the total caloric intake of both groups was nearly the same (Fig. 1E). However, the amount of calories consumed from fat source was significantly higher in the group fed an HFD than in the group fed an LFD (i.e., a 6-fold of difference; Fig. 1E).

In addition, we also evaluated the prostate weight and some relevant proliferation markers (i.e., Ki67, Men1, Ptg, c-Myc, and p27), which could serve as surrogate markers of hyperplasia, to evaluate the impact of diet and metformin treatment on this gland (Fig. 2). Remarkably, prostate glands did not exhibit changes in average weight at the end of the experiment among the different groups (Fig. 2A). However, prostates from mice included in the HFD-H2O group presented a significant increase in some of the proliferation markers analyzed (Ki67 and Men1 but not Ptg, p27, and c-Myc) mRNA levels when compared with LFD-H2O. Interestingly, this difference was not patent when comparing HFD-Met with LFD-Met groups (Fig. 2B).
Metformin reduced tumor growth, necrosis, and mitosis in a diet-dependent manner

The analysis of tumor growth in the different groups revealed that HFD had a significant effect on tumor growth, as tumor volume was significantly higher in HFD-H2O than in LFD-H2O mice (Fig. 3A and B). Remarkably, metformin treatment showed a clear antitumoral effect in both LFD-Met and HFD-Met groups compared with their controls (LFD-H2O and HFD-H2O, respectively), being more pronounced in the HFD-Met group (Fig. 3A and B). Indeed, in LFD-Met versus LFD-H2O groups, statistical differences in tumor volume were not found until 35 days after metformin treatment (56 days after prostate cancer cells inoculation), which were maintained until the day of euthanasia (Fig. 3A and B). In contrast, in HFD-Met vs. HFD-H2O groups, tumor volume differences appeared 22 days after starting metformin treatment (43 days after prostate cancer cells inoculation) and maintained statistical differences until the end of the study (Fig. 3A and B). Interestingly, the HFD-Met group exhibited the smaller tumor among all the groups at the end of the study (Fig. 3A and B).

Consistently, tumor weights at euthanasia were significantly lower in the HFD-Met group than in HFD-H2O and LFD-Met groups (Fig. 3C).

After histologic analysis, all tumors were classified as Gleason 10 poorly differentiated adenocarcinoma, indicating that all the tumors generated presented similar histotypes (Supplementary Fig. S1). However, metformin treatment clearly affected tumor necrosis and mitosis rate in a diet-dependent manner (Fig. 3D). Indeed, LFD groups exhibited similar necrosis and mitosis rate irrespective of the treatment, whereas metformin significantly reduced tumor necrosis but also mitotic index under HFD conditions (HFD-Met vs. HFD-H2O; Fig. 3D).

Metformin regulated specific tumor-associated pathways in a diet-dependent manner

To unveil the molecular alterations associated to the effect of diet and metformin on tumor growth, an expression array that comprises the examination of the expression levels of 730 mRNA implicated in 13 key cancer-related pathways was implemented in tumoral samples from the 4 experimental groups (see specific changes between groups in Supplementary Tables S3–S6). This analysis demonstrated a close interaction between diet and metformin in the regulation of several tumor-associated genes (Fig. 4A).

First, HFD-H2O promoted significant changes in the expression of 12 genes associated with MAPK, TGFβ, GnRH, and sphingolipid pathways compared with tumors formed in LFD-H2O group (Fig. 4A and B; Supplementary Fig. S2A and Table S3). Second, under LFD conditions, metformin treatment was associated with the alteration of 9 genes associated to Ras, MAPK, endocrine resistance, and EGFR pathways compared with LFD-H2O (Fig. 4A and C; Supplementary Fig. S2B and Table S4).

Finally, metformin induced a profound dysregulation of tumor-related genes under HFD conditions. Indeed, tumors induced in the HFD-Met group presented 120 genes differently expressed when compared with HFD-H2O (Fig. 4A; Supplementary Table S5) and 136 when compared with LFD-Met group tumors (Fig. 4A; Supplementary Table S6). In both cases, we found commonly altered pathways associated to metformin treatment under HFD conditions such as PI3K/Akt, MAPK, Jak/STAT, apoptosis, P53, cell cycle, TNF, prostate cancer, proteoglycans in cancer and insulin signaling and resistance signaling pathways [Fig. 4D (HFD-Met vs. HFD-H2O) and E (HFD-Met vs. LFD-Met)]. However, some pathways were exclusively altered in HFD-Met versus HFD-H2O such as mTOR, TGFβ, AMPK, and...
and CCND2, GADD45G, in this case, the most relevant changes were the upregulation of genes could cluster both groups separately (Fig. 4F; bottom).

– found that JAK/STAT and, once again, cell-cycle associated to reduced cell-cycle progression (Supplementary Figs. S5A and S6), the upregulation of the JAK/STAT pathway (Supplementary Figs. S5B and S6).

As diet-induced metabolic dysbalances and tumor growth can be associated with alterations in the components of the GH/IGF axis (25), we determined plasma Gh and Igf1 levels and the tumoral expression of GHR, insulin receptor (INSR), insulin-like growth factor 1 receptor (IGF1R), and IGF1-binding protein 3 (IGFBP3) in all groups (Fig. 5). Our data indicated that although circulating Gh and Igf1 levels did not significantly change by diet or treatment, metformin seemed to increase Gh levels (P = 0.18 by 2-way ANOVA, Fig. 5A) and to reduce Igf1 levels (P = 0.09 by 2-way ANOVA, Fig. 5A). The expression of some of these receptors and IGFBP3 was altered by metformin treatment and/or diet. Specifically, HFD increased GHR, IGFBP3...
and decreased INSR (Fig. 5B). Interestingly, metformin increased GHR expression under LFD conditions but decreased under HFD feeding (Fig. 5B). Moreover, IGFR was downregulated by metformin treatment only in the LFD group (Fig. 5B), and IGFBP3 expression was downregulated by metformin in the HFD group (Fig. 5B). In contrast, metformin treatment did not alter INSR.
expression levels (Fig. 5B). Next, as mRNA gene expression does not always necessarily reflect changes in the protein expression levels, we aimed to validate key results by Western blotting. However, because of the fact that available specific commercial antibodies against these proteins (GHR, INSR, IGF1R, and IGFBP3) are limited and their validity is compromised [as we have recently reported in a previous study (ref. 26)], together with the limited amount of protein obtained from each tumor, led us to evaluate AKT phosphorylation as a marker of the activation of the referred receptors. Despite the observed changes on the upstream signaling at mRNA levels, we did not find significant changes in the p-AKT/AKT ratio between experimental groups (Fig. 5C).

Figure 5.
Effects of diet and metformin on the components of GH, IGF1, and insulin axis. Circulating Gh and Igf1 levels of LFD- or HFD-fed mice, inoculated with PC3 cells and treated with vehicle or metformin (Met; 250 mg/kg/d in drinking water) at euthanasia (n = 5–6 mice; A). mRNA expression levels of GHR, INSR, IGF1R, and IGFBP3 (absolute mRNA copy number adjusted by a normalization factor (NF) calculated from the expression of HPRT and β-ACTIN) in PC3 xenografted samples (n = 9–11 tumors; B). AKT phosphorylation status on the different tumor samples determined by Western blotting (C). Values represent the mean ± SEM. Asterisks (*, P < 0.05) indicate significant differences compared with the respective control.
However, it should be mentioned that there existed a tendency for the reduction of AKT signaling in metformin-treated animals compared with HFD groups ($P = 0.07$), which did not reach statistical significance probably due to the limited number of samples available ($n = 5–6$/group).

**Cell line proliferation and migration**

To corroborate the direct effect of metformin on prostate cells, we analyzed the effect of metformin on key functional parameters (proliferation, migration, and PSA secretion) using tumoral and normal human prostate cell lines. First, we observed that the inhibitory effect of metformin treatment on proliferation rate seemed to be time-, dose-, and cell line–dependent. Particularly, metformin (2.5–5 mmol/L) decreased the proliferation of normal RWPE1 cells at 72 hours of incubation (Fig. 6A). Similar results were observed in LNCaP cells, as metformin treatment decreased the proliferation rate at the different doses tested (1, 2.5, and 5 mmol/L), whereas metformin only decreased proliferation in PC3 cells after 72 hours of incubation with a 5 mmol/L dose (Fig. 6A). However, metformin did not affect proliferation rate of 22Rv1 cells at any dose or time of incubation tested (Fig. 6A). Furthermore, metformin treatment (5 mmol/L) also decreased cell migration in PC3 cells (~30%) and in normal RWPE1 cells (~80%; Fig. 6B). However, metformin did not alter PSA secretion in LNCaP cells but tended to decrease PSA secretion in 22Rv1 cells ($P = 0.06$; Fig. 6C).

**Discussion**

Prostate cancer, one of the most severe health problems for the male population worldwide, is strongly influenced by...
obesity, wherein metformin could represent a promising treat-
ment (12). However, the endocrine metabolic, cellular, and
molecular mechanisms underlying these associations are still
unknown. In this study, we demonstrate, for the first time, that
HFD is associated with enhanced prostate cancer growth irre-
spective of body weight gain and endocrine metabolic dysre-
gulations and that metformin can reduce prostate cancer
growth under LFD but more prominently under HFD, acting
through the modulation of several key tumoral-associated
processes such as cell-cycle regulation, apoptosis, etc. It should
be, however, noted that all these data have been generated from
human prostate cancer cell line–derived tumors engrafted on
immunodeficient mice fed different diet regimens and that,
therefore, the results must be carefully translated to other
prostate cancer and diet models.

To determine the relative contribution of the obesity-associ-
ated dysregulations of the endocrine metabolic milieu (altera-
tions in the glucose/insulin metabolism and other systemic
changes) in the pathologic association between HFD and pros-
tate cancer and in the beneficial antitumoral effects of metfor-
min, we used a mouse model with partial resistance to HFD-
induced obesity (16–18) to be able to analyze the role of the
intake of higher fat proportion in the diet (HFD-feeding) and
metformin treatment in the progression of xenografted prostate
cancer cells without the confounding effects of dysregulated
endocrine metabolic conditions. As previously reported, this
mouse model did not significantly gain body weight under HFD
feeding compared with LFD animals (16–18), which was ac-
companied by similar body composition and glucose, insulin,
and corticosterone levels, with the only observed alteration in
leptin levels, which were elevated in HFD-fed mice. Moreover,
although we did not specifically analyze the level of hyperplasia
in the prostate in response to HFD, which has been previously
associated with obesity (5), we found that various surrogate
proliferation markers were upregulated (i.e., Ki67 and Men1)
under HFD conditions. Under these conditions, tumors en-
grafted in HFD-fed animals exhibited an accelerated growth
rate compared with LFD-fed mice, suggesting an association
between high calorie intake from fat and prostate cancer growth.
Of note, these results are consistent with previous studies in
TRAMP mice showing that HFD can promote prostate tumor
formation, likely due to an increase in cytokines (27), and
tumor growth compared with lean mice (28). Similarly, these
data are also in agreement with previous reports showing that
an HFD could increase the tumor growth in LNCAp xenografts,
wherein this effect was due to high levels of diet-induced
insulin/IGF1 (29, 30). However, this is the first study demon-
strating that the intake of a higher fat proportion in the diet due
to HFD feeding can promote the growth of the xenografted
prostate cancer cell irrespective of major metabolic dysregula-
tions including body composition and glucose, insulin or, even,
corticosterone levels. Indeed, we performed a gene expression
array in a subset of the tumors formed which indicated that
these effects may be due to the modulation of certain genes
involved in relevant tumor-associated pathways as is the case
of increased expression of MAP2K6 and PLCG1 or decreased
expression of SMAD4 and PRKAR1B, which have been shown
to be involved in the activation of MAPK or GHRH pathways
(31, 32). It should be nevertheless noted that these mice
presented elevated leptin levels under HFD feeding, which have
been associated with JAK/STAT pathway activation and tumor
growth (33) and could, therefore, help explain, at least in part,
the results observed herein. It is also worth mentioning that
these mice were young (i.e., 19 weeks of age at the end of the
study) and, at these doses used, it has not been demonstrated the
mechanisms underlying the antitumoral action of metformin in vivo and whether metfor-
min could also exert beneficial effects on prostate cancer develop-
ment and progression under normal metabolic conditions. In
particular, the data presented herein confirm and expand previous
results demonstrating a clear antitumoral role of metformin on
prostate cancer cells. However, although previous studies showed
similar effects of metformin on the regulation of diverse cell lines
(20), we found different responses to metformin depending on
cell type, dose, and treatment duration. Particularly, we observed
that 22Rv1 cells were not affected by metformin at any dose or
time tested, whereas metformin exerted clear effects on LNCAp
and PC3 cells. Indeed, the LNCAp cells seemed to be the most
sensitive, showing decreased proliferation after 48 hours with 2.5
to 5 mmol/L of metformin, whereas PC3 cells presented signifi-
cantly lower proliferation only after 72 hours with 5 mmol/L of
metformin. Interestingly, the behavior of the different cell lines
to metformin could be related to the PTEN expression considering
that both LNCAp and PC3 have a normal PTEN status whereas
22Rv1 does not, together with the fact that P13K/PTEN status seems
to be important for diet and GH/IGF1-mediated signaling (35, 36).
In addition, metformin also decreased prolif-
eration in normal RWPE1 cells at 72 hours at the higher doses
(2.5–5 mmol/L). Furthermore, 5 mmol/L metformin treatment
also decreased migration of PC3 and RWPE1 cells. Although,
metformin does not affect PSA secretion in LNCAp cells, it seemed
to decrease it in 22Rv1 cells ($P = 0.06$). Therefore, these results
suggest a differential role of metformin on functional para-
eters associated with the pathophysiology of the prostate gland;
however, it is worth mentioning that these results have been
generated by using normal and prostate cancer cell lines and
therefore, caution should be taken when trying to translate these
results to in vivo prostate gland behavior. Moreover, it should
be mentioned that although the doses used in vitro and in vivo
are not identical, these doses have been selected on the basis of
previous studies that demonstrate that higher doses of met-
formin are required in vitro to elicit the effects observed in vivo
(for review, see ref. 37).

In this context, it is even more noteworthy the fact that our
results demonstrate an effective antitumoral role of orally con-
sumed metformin under both, LFD and HFD, conditions. In
the case of LFD feeding, the tumors induced in mice treated
with metformin exhibited reduced volume and growth than those
induced in control (H2O)-treated mice, demonstrating that
metformin could also display antitumoral effects on prostate
cancer development and progression in vivo under conditions of
standard nutrition and balanced endocrine metabolic status, through
the modulation of the expression of key genes (DAXX, ERBB2, or
SHC4) involved in tumor-associated pathways such as MAPK or
EGFR. These data correlate well with previous studies reporting
that intraperitoneally injected or oral metformin treatment has
the ability to reduce tumor volume in LNCaP xenografts under chow diet (13) and that different concentrations of intraperitoneally injected metformin could reduce PC3-generated tumors in a dose-dependent manner on mice fed a normal diet (20).

As expected, our data demonstrate that metformin especially exhibited antitumoral effects on prostate cancer cells under HFD conditions, which is consistent with recent studies (20), reporting that metformin treatment reduces tumor growth after 4 weeks of a daily intraperitoneal injection of 250 mg/kg in HFD-fed mice. However, our results demonstrate for first time that, surprisingly, metformin treatment is much more effective under HFD conditions than under LFD, despite the fact that HFD-fed animals did not present severe endocrine metabolic dysregulation, which suggests a putative crosstalk between HFD and tumor development and progression. This improved performance of metformin on prostate cancer cells under HFD conditions was associated with the modulation of the expression of numerous genes involved in the pathologic development or progression of the tumors. Indeed, the molecular analysis implemented on tumors formed in HFD-fed mice treated with metformin revealed a drastic alteration on the expression of genes associated with several pathways and cellular processes including apoptosis, cell cycle, Jak/Stat, PI3K/Akt, MAPK, p53, TNF, insulin signaling and resistance, TGFβ signaling pathways (when comparing HFD-Met vs. HFD-H2O or LFD-Met mice) and mTOR, AMPK, and NF-κB (which were exclusively altered in HFD-Met vs. HFD-H2O mice). In this regard, it is remarkable the fact that the expression pattern of some of these genes involved in certain pathways and cellular processes such as apoptosis, Jak/Stat, and specially cell cycle [(upregulation of TNF, NGF, CCND2, IL12A and down-regulation of CBLC (Cbl proto-oncogene C)] can discriminate between the tumors induced in HFD-Met mice compared with the tumors formed in HFD-H2O or LFD-Met mice, suggesting that metformin could induce a characteristic gene expression fingerprint associated with cell-cycle progression under HFD, which could be associated with its antitumoral effects under HFD conditions (38–43). Moreover, these results are consistent with the anatomicopathologic analysis of the samples, which indicates that HFD-Met mice tumors exhibited lower number of mitosis than HFD-H2O mice.

Finally, although HFD-fed mice did not exhibit drastic changes in the endocrine metabolic profile (likely due to the particular resistance of this strain), obesity and diet have been associated in many studies with a dysregulation of the regulatory GH/IGF1 axis (26). This could be of particular importance considering the role exerted by this regulatory axis on the development and progression of prostate cancer. In particular, high IGF1 levels are related with higher incidence, increased progression, and poor prognosis of several human cancers (44), wherein men with high circulating IGF1 concentrations have been shown to exhibit 47% higher risk of developing prostate cancer (44). Indeed, IGF1 is an important regulator of cell proliferation, differentiation, and apoptosis (45), and the GH/IGF1 axis has been shown to be associated with prostate cancer development (46). Interestingly, we found a slight tendency for the reduction of IGF1 levels under metformin treatment irrespective of the diet ($P < 0.1$), which could help explain, at least in part, the fact that metformin-treated mice presented smaller tumors. In addition, we observed that IGFI R, which has been associated with tumor growth or progression and is over-expressed in prostate cancer (47), was decreased in LFD-Met mice, which is in line with a previous report (19) and provides additional putative drivers of the beneficial effects elicited by metformin under LFD conditions. On the other hand, metformin treatment in HFD-fed mice was associated with decreased expression of GHR and IGFBP3. Interestingly, GHR appeared to be upregulated in colorectal and breast cancer samples (48), and its downregulation is associated with better metabolic profile and lower cancer incidence (49), whereas IGFBP3 levels are elevated in patients with prostate cancer (50), suggesting that metformin could be partially exerting its beneficial effects under HFD conditions through the regulation of the local expression of different GH/IGF1 axis components. Remarkably, whether these expression changes are accompanied by alterations in protein levels is still to be defined.

Altogether, our results demonstrate that HFD is associated with enhanced prostate cancer growth in xenografted tumors irrespective of body weight gain and endocrine metabolic dysregulations and that metformin is able to reduce prostate cancer growth under LFD but more prominently under HFD, acting through the modulation of several key tumoral-associated processes such as cell cycle and/or apoptosis. Interestingly, we also observed that metformin had dissimilar effects on proliferation, migration, and PSA secretion from different cell lines and that some of the actions observed in vivo could be mediated by the modulation of the local expression of GH/IGF1 axis components.
References


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