Inositol Hexaphosphate Inhibits Tumor Growth, Vascularity, and Metabolism in TRAMP Mice: A Multiparametric Magnetic Resonance Study

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Abstract
Herein, employing anatomical and dynamic contrast-enhanced (DCE) magnetic resonance imaging (MRI), we evaluated noninvasively, the in vivo, chemopreventive efficacy of inositol hexaphosphate (IP6), a major constituent of high-fiber diets, against prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Male TRAMP mice, beginning at 4 weeks of age, were fed with 1%, 2%, or 4% (w/v) IP6 in drinking water or only drinking water till 28 weeks of age and monitored using MRI over the course of study. Longitudinal assessment of prostate volumes by conventional MRI and tumor vascularity by gadolinium-based DCE-MRI showed a profound reduction in tumor size, partly due to antiangiogenic effects by IP6 treatment. As potential mechanisms of IP6 efficacy, decrease in the expression of glucose transporter GLUT-4 protein together with an increase in levels of phospho-AMP-activated kinase (AMPKThr172) were observed in prostate tissues of mice from IP6 fed-groups, suggesting that IP6 is interfering with the metabolic events occurring in TRAMP prostate. Investigative metabolomics study using quantitative high-resolution 1H-NMR on prostate tissue extracts showed that IP6 significantly decreased glucose metabolism and membrane phospholipid synthesis, in addition to causing an increase in myoinositol levels in the prostate. Together, these findings show that oral IP6 supplement blocks growth and angiogenesis of prostate cancer in the TRAMP model in conjunction with metabolic events involved in tumor sustenance. This results in energy deprivation within the tumor, suggesting a practical and translational potential of IP6 treatment in suppressing growth and progression of prostate cancer in humans. Cancer Prev Res; 6(1); 40–50. ©2012 AACR.

Introduction
Prostate cancer is the most frequently diagnosed malignancy in elderly American men; however, its incidence varies dramatically on a global level; both incidence and associated mortality are lower in the Asian compared with Western countries (1). Importantly, the incidence of the occult/indolent form of PCa is similar globally despite dramatic difference in the occurrence of clinical malignancy (2). One possible explanation for this enigma could be the aging process, which inadvertently causes slow histopathologic changes in the prostate, while environmental/dietary factors serve as a trigger for the progression to more aggressive forms and are, thus, involved in the promotion rather than initiation of prostate cancer (2–4). In an effort to identify such etiologic factors, several studies indicate that 1 of the possible causes is the Western diet that includes highly processed foods (4–6). In the Asian diet, however, 1 essential component present ubiquitously is inositol hexaphosphate (IP6) or phytic acid, which is a naturally occurring hexaphosphorylated carbohydrate, present abundantly in diets with high-fiber content, most cereals, legumes, nuts, and soybean (5, 7–9). Importantly, IP6 is already marketed as a dietary supplement because of its antioxidant property and known beneficial effects, such as prevention of the formation of kidney stone, high cholesterol, and heart and liver diseases (5, 6, 8, 9).

The fact that the incidence of prostate cancer is lower in Asian men has triggered interest in IP6 as a possible factor in prostate cancer prevention (5, 9). However, due to its metal chelating properties, IP6 is often referred to as an antinutrient by the nutritionists, holding its dietary intake responsible for mineral deficiencies (10, 11). Contrary to this, several cancer researchers emphasize that IP6 manifests as an antinutrient only when the diet is already poor in trace elements, and strongly recommend IP6 intake for its
beneficial anticancer properties (6, 9, 12, 13). It is speculated that the anticancer effect of IP6 is mediated via its conversion to lower inositol phosphates which play essential roles in cellular signal transduction and regulation of cell growth and differentiation (6). Moreover, exogenously administered IP6 is quickly absorbed by the gastrointestinal tract and rapidly taken up by malignant cells (6, 14), thereby modulating their essential survival pathways; importantly, IP6 has no cytostatic or cytotoxic effect on normal cells and tissues (6). In this regard, several research groups, including ours, have shown the in vitro and in vivo anticancer efficacy of IP6 against a variety of cancers including prostate cancer (5, 6, 9, 15–22). Earlier mechanistic studies by us have shown that IP6 possesses strong anticancer efficacy against both androgen-dependent and -independent prostate cancer cell types, wherein, it inhibits cell growth, causes G1 cell-cycle arrest via modulation of cell-cycle regulatory molecules, and induces apoptotic death (16, 21). Other studies showed that IP6 impairs erbB1 receptor-associated mitogenic signaling and inhibits constitutive activation of NF-kB and induces apoptotic death (16, 21). Other studies showed that IP6 impairs erbB1 receptor-associated mitogenic signaling and inhibits constitutive activation of NF-kB and induces apoptotic death (16, 21).

Accordingly, in the present study, we fed different doses of IP6 in drinking water to 4-week-old TRAMP mice till 28 weeks of age, and assessed the inhibitory effect of IP6 on tumor growth, progression, and angiogenesis using longitudinal study parameters [employing conventional magnetic resonance imaging (MRI) and gadolinium-based dynamic contrast-enhanced (DCE-MRI)] as a criterion (25, 26) to simultaneously assess IP6 effect on prostate sizes/volume and tumor vascularization as a function of time, and to also elucidate the molecular events involved in IP6 efficacy. Furthermore, the metabolic activity in tumor tissues was also assessed by quantitative 1H-NMR metabolomics at the end of the study.

Materials and Methods

Animals and treatment

Heterozygous TRAMP (C57BL/6) females were crossbred with nontransgenic C57BL/6 breeder males, and the progeny subjected to genotyping for PB-Tag as previously described (23). The routinely obtained 4-week-old TRAMP male mice were randomly distributed into positive control and treatment groups. Positive control mice were supplied with regular drinking water and the treatment groups were fed 1%, 2%, and 4% (w/v) IP6 in regular drinking water for 24 weeks. For additional details on experimental design and drug doses, please see supplementary material.

Magnetic resonance imaging

We employed MRI (27, 28) (i) to assess prostate and tumor sizes in the mouse using fast spin-echo proton density-weighted MRI, and (ii) to noninvasively assess changes in tumor vascularization (perfusion and permeability) by gadolinium (Gd)-based DCE-MRI (fast-spin echo continuous T1-acquisitions with 60 second baseline followed by a bolus injection of 0.1 mmol/kg MultiHance via a tail catheter and 10 minutes of continuous MRI acquisition; ref. 28). Bruker 4.7 Tesla/16-cm MRI/MRS PharmaScan (Bruker Medical) with a mouse volume transmitter/receiver coil (36 mm diameter) was used for all MRI studies (at the Animal Imaging Shared Resources, University of Colorado Anschutz Medical Campus). All sequence parameters and image analysis were as previously described by Troiani and colleagues (27). Additional details are provided in supplementary materials and methods.

Necropsy and histopathology

At the time of sacrifice, mice were euthanized by carbon dioxide asphyxiation followed by exsanguination. Each mouse was weighed and the lower urogenital tract (LUT), including bladder, seminal vesicles, and prostate, was removed en bloc. The LUT wet weight was recorded, and prostate gland/tumor was harvested, microdissected, partly snap-frozen, and partly processed for histopathologic and immunohistochemical (IHC) analyses as previously described (29).

Quantitative 1H-NMR analyses

Snap-frozen prostate tumor tissues were extracted using 8% perchloric acid (Sigma-Aldrich) and analyzed by NMR as previously described (30, 31). All high-resolution 1H-NMR spectra were obtained at a Bruker 500 MHz DRX spectrometer (Bruker BioSpin) equipped with a standard 5-mm TXI probe, using 0.6 mmol/L trimethylsilylpropionic acid (TSP) as a chemical shift and concentration standard (30, 31). Absolute concentrations of 36 metabolites (normalized to the wet weights of each sample) were assessed and presented as micromoles per milligram of tissue (31, 32).

Statistical and microscopic analyses

All MRI and 1H-NMR analyses were conducted by the MR scientists (NJS and KMH) who were blinded to the group assignment of the animals/samples. For metabolomics, absolute individual concentrations of distinguished biomarkers were analyzed by ANOVA followed by Tukey post hoc test. The Fisher Exact test was used to compare the incidence of pathologic lesions in different groups. All other data were analyzed by the unpaired 2-tailed Student t test. The significance level was set at P values less than 0.05 for all tests (Sigma Plot-version 9.01, Systat Software, CA and SPSS version 14.0, SPSS Inc.).

Results

IP6 feeding reduces LUT weight without any apparent toxicity

IP6 feeding did not show any significant changes in fluid consumption between positive control and 1%- and 2%
IP6-fed mice during the entire study (data not shown). In the 4%–IP6-fed group, however, mice showed lower fluid consumption compared with other groups, which could be attributed to the taste of the highly concentrated IP6 solution. In addition, IP6 feeding did not show considerable difference in diet consumption and body weight-gain profiles between positive control and IP6-fed mice during the entire study (data not shown). At necropsy, all animals were examined for gross pathology, and there was no evidence of edema and abnormal organ size or appearance in non-target organs. It is important to emphasize here that, because earlier studies have reported that experimental rats fed with pure phytate for their life-time did not show any effect in serum or bone minerals (except for lower zinc concentration in bone of second-generation rats; refs. 6, 12, 13), we did not focus our efforts in determining the mineral bioavailability in the TRAMP mice in the present study. There was, however, a significant difference between the LUT weight (normalized to body weight) of positive control mice compared with the IP6-fed groups. The normalized LUT weight of 1%, 2%, and 4%–IP6-fed groups was 39% ($P < 0.001$), 62% ($P < 0.001$), and 53% ($P < 0.001$) lower than that of positive control group (Fig. 1A). In nontransgenic mice, IP6 feeding did not show any change in diet and fluid consumptions, and body weight-gain profiles (data not shown). In addition, no pathologic changes in the prostate or other organs were observed in nontransgenic mice in IP6-fed versus control groups (data not shown).

**IP6 feeding reduces prostate volume in TRAMP mice (MRI-study)**

Longitudinal assessment of prostac/tumor volumes from 4 weeks till 28 weeks of mice age was carried out in both positive control (untreated) and IP6-treated TRAMP mice ($n = 6$ per group), using noninvasive proton density-weighted MRI (Fig. 1B and C). The results indicated that the 2% to 4% IP6 feeding did not affect prostate volumes till 12 weeks of age (an initiation phase of the disease), but significantly decreased prostate volumes at 16 weeks of age till the end of the treatment (28 weeks of age) compared with untreated controls (Fig. 1B and C). While both 2% and 4% IP6 doses caused a decrease in prostate/tumor volumes during the progression stages (Fig. 1), the 1% IP6 dose had no significant effect compared with the positive control (data not shown).

**IP6 feeding inhibits progression of prostate cancer**

Using the classifications previously described (33, 34), the histopathologic analysis of the hematoxylin and eosin (H&E)-stained dorsolateral prostate showed that there was a marked difference in prostatic intraepithelial neoplasia (PIN) and adenocarcinoma incidences between positive control and IP6-fed groups (Fig. 2A and B).
increase in the incidence of more differentiated tumors in IP6-fed groups was also observed compared with positive controls, with a concomitant reduction in the incidence of more aggressive tumors in IP6-treated mice (Fig. 2B). Importantly, there was no incidence of poorly differentiated (PD) adenocarcinoma in both 2% and 4% IP6-fed groups compared with 56% incidence in positive controls. As shown in Fig. 2C, there was also a significant reduction in tumor grade in IP6-fed groups, which was calculated on the basis of criteria described previously (33). The distribution of PIN lesions also indicated that there was a significant increase in the area covered by LGPIN lesions in the mice fed 2% and 4% IP6 (Fig. 2D). More importantly, in 2%- and 4%-IP6-fed groups, approximately 3% to 6% area of prostate also displayed normal histology, which was not the case in both the 1% IP6 dose and positive control groups (Fig. 2D). In nontransgenic mice, prostate histopathology did not show any difference between control and the IP6-fed group (data not shown). Together, these results suggest that IP6 feeding is effective in decreasing the progression of preneoplastic lesions in the prostate of TRAMP mice to more aggressive forms of adenocarcinoma; however, these chemopreventive effects of IP6 do not seem to be dose dependent, with the group given the 2% IP6 dose performing better than the group given the highest dose. Because the lowest dose of IP6 (1% IP6-fed group) was unable to decrease the incidence and severity of prostatic lesions better than the other 2 higher doses, we decided to limit our further mechanistic investigations to the groups that were fed the higher doses of IP6.

**IP6 feeding decreases the proliferation index and increases apoptosis in the prostate of TRAMP mice**

The in vivo antiproliferative effect in both 2%- and 4%-IP6-fed groups was significantly higher than that in untreated mice, although the effect of 2% IP6 feeding was relatively better (P = 0.01) than the 4% IP6 dose (Supplementary Fig. S1A). With regard to the in vivo apoptotic effect, whereas both doses of IP6 increased apoptotic cells in TRAMP prostate tissue, it was again the 2%–IP6-fed group that showed a statistically significant proapoptotic effect as evidenced by a 4-fold (P = 0.01) increase in apoptotic cells (Supplementary Fig. S1B).

**IP6 feeding inhibits angiogenesis in TRAMP mice (DCEMRI and IHC study)**

Because the ability of localized carcinoma to advance to invasive stages is dependent on its ability to recruit new vasculature via angiogenesis (35), we also determined the effect of IP6 on tumor vasculature. In TRAMP mice, prostate vasculature undergoes a proangiogenic switch, with increase in tumor grade leading to higher expression of proangiogenic factors resulting in increased microvessel density (MVD), which further promotes progression to invasive stages (35, 36). In this regard, we observed that IP6 feeding significantly decreased tumor perfusion/permeability and MVD as indicated by DCE-MRI studies (on the basis of gadolinium uptake and kinetics in prostate tissues as an indicator of angiogenesis; Fig. 3A and B) and IHC staining for CD-31 (Supplementary Fig. S1C), respectively. IP6 treatment led to a dose-dependent decrease (up to 3-fold by 4% IP6) in gadolinium IAUCs (initial gadolinium uptake over time) in gadolinium IAUCs (initial gadolinium uptake over time) and initial gadolinium IAUCs (initial gadolinium uptake over time).
uptake in first 90 seconds after gadolinium injection), which is an indicator for decreased tumor perfusion. The universal parameters for tumor vascularity (total AUC; the volume transfer constants, K<sub>trans</sub>; and the extravascular fractions, V<sub>e</sub>) were similarly decreased in the IP6-fed groups. These results indicated that IP6 might be exerting its antiangiogenic effect by affecting the expression of proangiogenic factors. IHC studies were done to support this assumption and to corroborate imaging results, where the expression of the proangiogenic factor VEGF (35, 37) was significantly decreased in IP6-fed mice compared with controls (P < 0.001, for both doses; Fig. 4A). Additional IHC studies were also carried to determine whether IP6 feeding affects the expression of inducible nitric oxide synthase (iNOS), an enzyme involved in the production of nitric oxide (NO) which facilitates neovascularization and invasion (38). The data indicated that IP6 significantly decreases (31%–34%, P < 0.001 for both doses) iNOS immunoreactivity scores (Fig. 4B). As transcription factor NF-κB mediates transcription of genes which are associated with cancer initiation and progression, including those encoding for VEGF and iNOS (39), we next assessed whether IP6 interferes with the expression of these proteins via downregulation of NF-κB activity. The IHC analysis showed that IP6 does inhibit NF-κB activity as evidenced by a significant decrease in the nuclear expression of phospho NF-κB/p65 in the TRAMP prostate of both doses of IP6-fed mice compared with controls (Fig. 4C). Additional mechanistic studies delineating the antiangiogenic effect of IP6 revealed that its activity was transduced by an upstream molecule, AKT, which is known to regulate NF-κB (40). Specifically, both 2% and 4% IP6-fed groups of mice showed a significantly reduced phospho AKT levels in IHC staining in the TRAMP prostate (Fig. 4D). Together, these results suggested that IP6 feeding inhibits the recruitment of new vascular network during angiogenesis, by
downregulating the expression of proangiogenic factors via a series of molecular events which, in totality, acted as a limiting factor that in turn restricted the ability of localized PIN/carcinoma to advance to a more invasive stage.

**IP6 feeding interferes with the expression of molecules associated with tumor sustenance and glucose transportation in TRAMP prostate**

Additional IHC studies were next conducted to examine the IP6 effect on the expression of transmembrane glucose transporter (GLUT) proteins, which mediate glucose uptake in the cells and thereby play an essential role in the first step of the glucose use cascade (41). While there was no change in GLUT-1 (data not shown), the expression of GLUT-4 protein (both membrane and cytoplasmic) was significantly decreased (57% and 34%, \( P < 0.001 \), for both doses) in the prostate of 2%- and 4%-IP6-fed mice compared with the positive control (Fig. 5A); the 2%-IP6 dose produced a significantly \( (P < 0.01) \) stronger effect than the 4% dose. The observed decrease in GLUT-4 protein levels suggested the potential of IP6 to decrease/limit the amount of glucose being pumped into the cells, which led us to predict that this decreased glucose uptake in the prostate of IP6-fed mice could result in a cellular stress associated with decreased ATP levels and increased AMP levels in the prostate (42). Further IHC studies were carried out to confirm this mechanistic assumption, which revealed that, indeed, there was an increase in the phosphorylated levels of AMP-activated kinase (AMPK\(_{\text{Th172}}\); Fig. 5B). Specifically, the immunoreactivity scores for phospho-AMPK\(_{\text{Th172}}\) and its downstream...
target, phospho ACC (42, 43), were increased by approximately 2-fold \( P < 0.05, P < 0.01, \) and \( P < 0.01, \) for both doses, respectively in the prostate of IP6-fed mice compared with the positive controls (Fig. 5). Together, these findings suggested that IP6 was interfering with the metabolic events occurring in the TRAMP prostate tumor tissue, which might have an important role in its observed chemopreventive efficacy.

### IP6 feeding causes metabolic alterations in the TRAMP prostate as assessed by \(^1\)H-NMRS

To further confirm that IP6 feeding was indeed interfering with the metabolic events involved in TRAMP prostate malignancy (31), we subjected the prostate tissues to a metabolomics study using \(^1\)H-NMRS (Table 1). A variety of cancerous tissues, including that of the prostate, exhibit altered metabolic profiles of choline-containing metabolites, specifically phosphatidylcholine (PtdCho) – the major phospholipid in the cell membrane – which is reported to be increased in a variety of tumors (44, 45). Our results indicated that IP6 feeding decreased the levels of all membrane phospholipids including PtdCho and phosphatidylinositol (PtdIns, both \( P < 0.04 \)) indicating a specific inhibition of cell membrane biosynthesis in the prostate (Table 1). Interestingly, the phospholipid precursors for PtdCho and PtdIns in the aqueous fraction were increased after IP6 feeding (phosphocholine and glycerophosphocholine, \( P = 0.001, \) as well as myoinositol, \( P = 0.02 \)). One of the most striking differences between the positive control and IP6-fed group was the accumulation of fatty acids (especially monounsaturated fatty acids) and a decrease in cholesterol. Accumulation of lipids, while accompanied by a significant decrease in phospholipid levels, is another confirmation for deterioration in cell membranes. While normal prostate glands express relatively high concentrations of polyols compared with prostate cancer lesions (46), it was interesting to observe increased concentrations of osmolyte myoinositol and other polyols and sugars in the IP6-fed group that are significantly decreased during prostate tumor progression (Table 1). Furthermore, although glucose was present in prostate tissues, the levels were quite low, below the NMR limit of quantification, in both IP6-fed and positive control mice. In our study, a decrease in lactate and alanine (end-products of glycolysis) was also observed in the IP6-fed group compared with controls (Table 1). Furthermore, IP6 feeding caused an increase in the levels of glutathione (the antioxidant glutathione serves as a free-radical scavenger) and decreased PUFA/MUFA ratios,
indicating an overall improved antioxidant defense and decreased necrotic fraction (32, 45) by IP6 feeding (Table 1).

Discussion

Herein, we applied multiparametric MRI on TRAMP mouse prostate consisting of a combination of anatomical proton density-weighted imaging for longitudinal tumor growth and DCE-MRI for tumor perfusion and permeability, to evaluate the IP6 effect on prostate tumorigenesis as a function of time. This noninvasive imaging technique showed that 1% IP6 dose was not significantly effective, but 2% and 4% IP6 doses showed a significant decrease in prostate volume after 16 weeks of mouse age, although they were ineffective in inhibiting tumorigenesis before this time.
point. These results suggest that IP6 does not interfere with PIN development, an initial stage of prostate tumorigenesis (23, 24). However, our results highlight the potential of IP6 to interfere with the events involved in the transformation of neoplastic lesions to more advanced forms of the disease that are initiated with increasing age of TRAMP mice (3, 29, 35) and cause the prostate to endure an insult of proangiogenic events (24, 29, 35). Histopathologic evaluation further corroborated the MRI imaging findings where IP6 had no inhibitory effect on PIN formation but decreased the formation of adenocarcinoma lesions. In fact, a higher incidence of PIN lesions and more differentiated tumors were observed in TRAMP prostate fed with 2% and 4% IP6. Overall, while we expected IP6 to display a dose-dependant efficacy at 1%, 2%, and 4% doses, the results showed that 2% IP6 was an optimum dose exhibiting significant anti-tumor efficacy. Notably, the 4% IP6 dose, although not toxic, was less effective than the 2% dose. To address this discrepancy, because IP6 feeding was done in drinking water, first we analyzed the fluid consumption data of different treatment groups, and found that the mice in the 4% IP6 group consumed the least water (data not shown). After correction for water consumption and IP6 concentration, the 4%–IP6-fed group of mice actually consumed even less IP6 than the 2%–IP6-fed group, which supports the IP6 efficacy outcomes as optimum at the 2% dose observed by us in this study.

With regard to our imaging approach, while volumetric assessment by MRI has been used as a "gold-standard" for treatment response to chemotherapeutic agents, it falls short in explaining the mechanisms of action of novel targeted therapies. A novel functional imaging DCE-MRI technique was applied in this study for noninvasive assessment of tumor angiogenesis. This analysis showed that IP6 feeding inhibits the development of new vasculature in TRAMP prostate which was later corroborated by IHC staining for CD-31. As a pertinent mechanism, IP6 was depleted, which in turn activates the mitochondrial death cascade leading to apoptotic death (50). Interestingly, IP6 feeding showed a statistically significant proapoptotic effect and phospho-ACC by IP6 feeding in the prostatic tissues. Indeed, in support of this assumption, the levels of lactate and alanine (end products of glycolysis) were reduced in IP6-treated prostatic tissues compared with the untreated positive control group.

Limited source of metabolic energy activates AMPK due to altered AMP to ATP ratio, which could alter the activity of ACC, a precursor for fatty acid synthesis (42). We observed increased levels of both phospho-AMPK (activated AMPK) and phospho-ACC by IP6 feeding in the prostatic tissues. These results are important, as in vitro studies by different research studies translating the effect of limited glucose supply on normal and malignant cells have shown that glucose withdrawal leads to cellular death in tumors through distinct mechanisms (50). Specifically, in some tumors, the mechanism of cell death seems to be ATP depletion, which in turn activates the mitochondrial death cascade leading to apoptotic death (50). Interestingly, IP6 feeding showed a statistically significant proapoptotic effect in TRAMP prostate tissue. Furthermore, the NMR metabolomics study revealed that although the levels of fatty acids were increased in the prostate tissue from IP6-fed mice (possibly due to an increase in cell membrane degradation and accumulation of fatty acids and lipids), a significant decrease was evident in cell membrane phospholipids (PtdCho and PtdIns) as an indicator of decreased cell membrane biosynthesis (decreased proliferation rates). In addition, a significant increase in total glutathione levels in the prostatic tissue of IP6-fed mice was observed,
suggesting that IP6 inhibits prostate cancer progression also in part by increasing antioxidant glutathione levels, which serves as a free-radical scavenger. Another striking effect of IP6-feeding was increased levels of myo-inositol; its decreased expression is reported with increased malignancy in prostate (46).

In summary, our results are both novel and highly significant in establishing that IP6 suppresses growth and progression of prostate cancer via its ability to alter tumor vascularity and the energy-generating metabolic events in the tumor cells. Because these mechanistic events eventually result in an arrest of tumor grade at neoplastic stages, the observed chemopreventive effect of IP6 against prostate cancer could have translational potential in controlling the clinical progression of prostate cancer in patients diagnosed early at the PIN stage of the disease.

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

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