

Research Article

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Metformin Prevents Tobacco Carcinogen–Induced Lung Tumorigenesis

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Abstract

Activation of the mammalian target of rapamycin (mTOR) pathway is an important and early event in tobacco carcinogen–induced lung tumorigenesis, and therapies that target mTOR could be effective in the prevention or treatment of lung cancer. The biguanide metformin, which is widely prescribed for the treatment of type II diabetes, might be a good candidate for lung cancer chemoprevention because it activates AMP-activated protein kinase (AMPK), which can inhibit the mTOR pathway. To test this, A/J mice were treated with oral metformin after exposure to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Metformin reduced lung tumor burden by up to 53% at steady-state plasma concentrations that are achievable in humans. mTOR was inhibited in lung tumors but only modestly. To test whether intraperitoneal administration of metformin might improve mTOR inhibition, we injected mice and assessed biomarkers in liver and lung tissues. Plasma levels of metformin were significantly higher after injection than oral administration. In liver tissue, metformin activated AMPK and inhibited mTOR. In lung tissue, metformin did not activate AMPK but inhibited phosphorylation of insulin-like growth factor-I receptor/insulin receptor (IGF-1R/IR), Akt, extracellular signal–regulated kinase (ERK), and mTOR. This suggested that metformin indirectly inhibited mTOR in lung tissue by decreasing activation of insulin-like growth factor-I receptor/insulin receptor and Akt upstream of mTOR. Based on these data, we repeated the NNK–induced lung tumorigenesis study using intraperitoneal administration of metformin. Metformin decreased tumor burden by 72%, which correlated with decreased cellular proliferation and marked inhibition of mTOR in tumors. These studies show that metformin prevents tobacco carcinogen–induced lung tumorigenesis and support clinical testing of metformin as a chemopreventive agent. *Cancer Prev Res*; 3(9); 1066–76. ©2010 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death in both men and women in the United States, and new targets are needed for the prevention and treatment of lung cancer. The serine-threonine kinase mammalian target of rapamycin (mTOR) could be a good target because it is an important regulator of processes critical to tumorigenesis, such as cellular metabolism, growth, and proliferation. Many cancers, including lung cancer, are characterized by aberrant activation of mTOR (reviewed in ref. 1).

Although activating mutations in mTOR itself have not been identified, deregulation of upstream components that regulate mTOR are prevalent in lung cancer. The prototypic mechanism of mTOR regulation in cells is through activation of the phosphatidylinositol 3-kinase/Akt pathway, but mTOR receives input from multiple signaling pathways, including the Liver Kinase β 1 (LKB1)/AMP-activated protein kinase (AMPK) pathway (reviewed in ref. 2).

Exposure of A/J mice to the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces K-ras–mediated lung tumorigenesis (3). Lung tumors that develop in these mice are characterized by increased activation of the mTOR pathway, and treatment with the mTOR inhibitor rapamycin prevents lung tumorigenesis in this model by 90% (4). Prevention of NNK-induced lung tumorigenesis by rapamycin is associated with inhibition of the mTOR pathway in tumor cells and with decreased lung- and tumor-associated regulatory T cells (Treg; ref. 5), which can suppress lung immunity and create a permissive environment for tumor development (reviewed in ref. 6). This suggests that rapamycin could be an effective chemopreventive agent in patients at high risk of developing lung cancer. However, the development and

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application of rapamycin as a chemopreventive agent may be limited because of toxicities. The identification of other drugs inhibiting mTOR that are well tolerated could increase opportunities for lung cancer chemoprevention.

One candidate is the biguanide metformin, which is the most widely prescribed drug for the treatment of type II diabetes. Long-term treatment is associated with few adverse effects (7). Metformin is an effective antidiabetic agent because it activates AMPK, a master regulator of cell metabolism (8–10). AMPK activation may also play a role in preventing the formation and progression of cancer because AMPK is activated by the tumor suppressor LKB1 (11). The LKB1/AMPK pathway is linked to inhibition of tumor growth through inhibition of the mTOR pathway (12). Interestingly, metformin use in humans is associated with a decrease in cancer incidence (13, 14), but whether this is related to activation of AMPK is unclear. Because AMPK inhibits the mTOR pathway, drugs that activate AMPK could be tested in the NNK-induced mouse model of lung tumorigenesis.

We investigated if two different routes of administration of metformin were effective in preventing NNK-induced lung tumorigenesis. Administration of oral metformin significantly prevented lung tumorigenesis by 40% to 50%, but only modestly inhibited the mTOR pathway in tumors. Intraperitoneal administration of metformin produced higher plasma concentrations of metformin, which increased the inhibition of the mTOR pathway in tumors and reduced lung tumorigenesis by ~75%. Inhibition of mTOR in lung tissue was surprisingly independent of AMPK and correlated with decreased circulating levels of insulin-like growth factor-I (IGF-I) and insulin, suggesting that chemoprevention of lung tumors by metformin was indirect. This mechanism of chemoprevention is similar to that observed in diabetics and provides an underpinning for the decreased cancer incidence associated with metformin use in diabetic patients.

Materials and Methods

Materials

The following antibodies were obtained from Cell Signaling Technologies: phospho-AMPK (T172), pan-AMPK α , phospho-acetyl-CoA carboxylase (ACC; S79), phospho-S6 (S235/236), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), phospho-IGF-I receptor/insulin receptor (IGF-1R/IR; Y1135/1146), phospho-Akt (T308), and phospho-extracellular signal-regulated kinase (ERK; T202/Y204). Antimouse/rat Foxp3 (clone FJK-15s) and CD3 antibodies were obtained from eBioscience and Dako, respectively. Ki-67 antibody was purchased from Vision Biosystems. NNK was obtained from EaglePicher Pharmaceuticals. Metformin, radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail, and phosphatase inhibitors were purchased from Sigma Chemicals. Recombinant mouse IGF-I and HUMULIN R were obtained from R&D Systems and Eli Lilly, respectively.

Tumorigenesis and biomarker studies in A/J mice

A/J mice were obtained from The Jackson Laboratory and housed according to the guidelines of the Animal Care and Use Committee of the NIH. For the tumorigenesis studies, 6-week-old female A/J mice were fed the AIN-93G diet (15, 16) and given three weekly injections of 100 mg/kg NNK, as previously described (4). One week following the last dose of NNK, mice were switched to the AIN-93M diet and randomized into a control group or groups that received 1 or 5 mg/mL metformin dissolved in drinking water ($n = 15$ mice per group). Mice were treated with this dosing schedule with metformin for 13 weeks, and their water was changed weekly. For the tumorigenesis study in which metformin was administered by intraperitoneal injection, mice were treated daily with saline or 250 mg/kg metformin beginning 1 week after the last dose of NNK ($n = 15$ mice per group). This dosing schedule with metformin was continued for 13 weeks. At the end of the tumorigenesis studies, mice were anesthetized using isoflurane, and blood was collected by cardiac puncture using BD vacutainer vials containing EDTA. Plasma was isolated by centrifugation of the blood samples and sent to the Laboratory of Proteomics and Analytical Technologies (Frederick, MD) for metformin pharmacokinetic analysis. Lung and liver tissues were also harvested, fixed in formalin overnight, and processed by Histoserv, Inc., for immunohistochemical analysis, or stained with H&E or Oil Red O. To determine tumor multiplicity, peripheral lung adenomas were counted using a dissecting microscope. Tumor volume was calculated by multiplying the three dimensions of each tumor. Two dimensions were measured using a micrometer, and the third dimension was estimated by averaging these dimensions. Tumor burden was calculated as the sum of individual lung tumor volumes in each mouse.

For the biomarker studies, 8-week-old female A/J mice were fed the tumorigenic diet AIN-93M and were injected with saline or 250 mg/kg metformin daily $\times 3$ ($n = 3$ mice per group). Mice were anesthetized with isoflurane 2 hours after the last injection of metformin, and liver or lung tissues were harvested and flash-frozen in liquid nitrogen. Because AMPK is exquisitely sensitive to the metabolic state of the animal, tissues were harvested early in the morning when the mice were in a "fed" state. Biomarker studies were performed at least three times.

Immunoblotting analysis

Frozen tissues were pulverized on dry ice, and lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. Immunoblotting analysis of tissue lysates was done as described previously (17). For immunoblotting analysis of p-AMPK or p-IGF-IR/IR, antibodies were used at a dilution of 1:500. All other antibodies were used at a dilution of 1:1,000. Densitometry was done using NIH Image software. To determine differences in the phosphorylation of 4E-BP1 using an antibody that recognizes total 4E-BP1, changes in the ratio of the top band (hyperphosphorylated 4E-BP1) to

the sum of the bottom two bands (hypophosphorylated 4E-BP1) were quantified.

Immunohistochemistry

Formalin-fixed lung tissues were paraffin-embedded, sectioned, and placed on slides (Histoserv). Immunohistochemical analysis was done on lung tissues from five mice per treatment group. Antigen retrieval was done using target retrieval solution (pH 6.0) and a decloaking chamber (Dako). Tissues were incubated in p-AMPK (1:50), p-S6 (1:100), Ki-67 (1:2,000), Foxp3 (1:25), or CD3 (1:600) antibodies per manufacturer's recommendations. To verify staining specificity, tissues were also incubated in the absence of primary antibody. Detection was done using VECTASTAIN Elite ABC kits (Vector Laboratories).

Immunohistochemical analysis for p-AMPK or p-S6 was quantified by assigning a score of absent (0), minimal (1), moderate (2), or high (3) staining to each tumor cell. All tumors in a single randomly selected section of lung tissue containing all lobes were scored. The staining index was calculated for each tumor by multiplying the staining intensity by its distribution. The staining indices were then averaged for all tumors in mice from each treatment group. Ki-67 staining was quantified by counting the number of Ki-67⁺ cells per tumor, and these values were averaged for all tumors in mice from each treatment group. Lung-associated percent Foxp3⁺/CD3⁺ cells were determined by averaging the number of Foxp3⁺ and CD3⁺ cells in ten 40× magnification fields containing normal lung parenchyma. Tumor-associated percent Foxp3⁺/CD3⁺ cells were determined by counting the number of Foxp3⁺ or CD3⁺ cells per tumor. Tumor-associated percent Foxp3⁺/CD3⁺ cells were then averaged for all tumors from mice in each treatment group. The investigator was blinded to the identities of the samples.

Pharmacokinetic analysis of metformin plasma levels

Sample preparation and analysis were done as previously described (18). Analysis was done on plasma collected from nine mice per treatment group at the end of the tumorigenesis study using oral administration of metformin, and from six mice injected with 250 mg/kg metformin intraperitoneal daily × 7, 2 hours after the last injection.

Quantitative reverse transcription-PCR for organic cation transporter 1

Lung and liver tissues were harvested from 10-week-old A/J mice ($n = 3$) and stored in RNAlater (Ambion). RNA was isolated from tissues using an electric homogenizer and the RNeasy kit (Qiagen). Quantitative reverse transcription-PCR (RT-PCR) for organic cation transporter 1 (OCT1) was done using a two-step process. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was subsequently done using SYBR Green PCR Master Mix and run on an ABI PRISM 7500 (Applied Biosystems). Expression of OCT1 mRNA was normalized to GAPDH to control for differences in

total mRNA between samples. Primer sequences for mouse OCT1 and GAPDH were used as previously published (19).

Blood glucose and plasma IGF-I and insulin analysis

Blood glucose levels were assessed in mice fed the tumorigenic diet AIN-93G/M or the cereal diet NIH-07 for 16 weeks. Mice were fasted for 4 hours before blood collection from the saphenous vein, and glucose levels were measured using a One Touch Ultra2 glucometer ($n = 5$ mice per diet). Plasma levels of IGF-I and insulin were measured using the Mouse/Rat IGF-I ELISA (Diagnostic Systems Laboratories) and the Rat/Mouse Insulin ELISA kit (Millipore), respectively. These analyses were done on plasma obtained from five mice per treatment group at the end of the tumorigenesis studies.

Statistical analyses

All analyses were done using a two-tailed Student's *t* test.

Results

Oral metformin significantly prevents NNK-induced lung tumorigenesis in A/J mice, but only modestly inhibits the mTOR pathway in lung tumors

To investigate if metformin prevents NNK-induced lung tumorigenesis, A/J mice were treated with 1 or 5 mg/mL metformin dissolved in drinking water for 13 weeks, beginning 1 week after administration of NNK (Fig. 1A). These doses of metformin were used because they have been previously shown to activate AMPK in multiple tissues in mice (20, 21). Metformin was well tolerated and did not significantly affect the weight of mice throughout the course of treatment (data not shown). To determine if oral metformin produced levels that could be achieved in humans, plasma concentrations were assessed at the end of 13 weeks. Mice that received metformin at 1 mg/mL achieved 0.45 µg/mL, and mice at 5 mg/mL achieved 1.70 µg/mL, which is consistent with steady-state values that have been reported in patients (0.5-2 µg/mL; Fig. 1B). Although metformin did not affect tumor incidence or pathology, lung tumor multiplicity was decreased by 28% (1 mg/mL) and 32% (5 mg/mL). Treatment with 5 mg/mL metformin also decreased average tumor volume by 34% (Fig. 1C, left and middle). Overall tumor burden was decreased by 39% (1 mg/mL) and 53% (5 mg/mL; Fig. 1C, right). To determine if oral metformin inhibited the mTOR pathway in lung tumors, immunohistochemical analysis was done using antibodies specific for the phosphorylated form of S6, a component downstream of mTOR. Oral metformin inhibited phospho-S6 by 20% in lung tumors (Fig. 1D). Taken together, these results show that oral metformin significantly prevented tumor growth, but to a lesser extent than rapamycin, which previously decreased lung tumorigenesis by 90% in this model system (4).

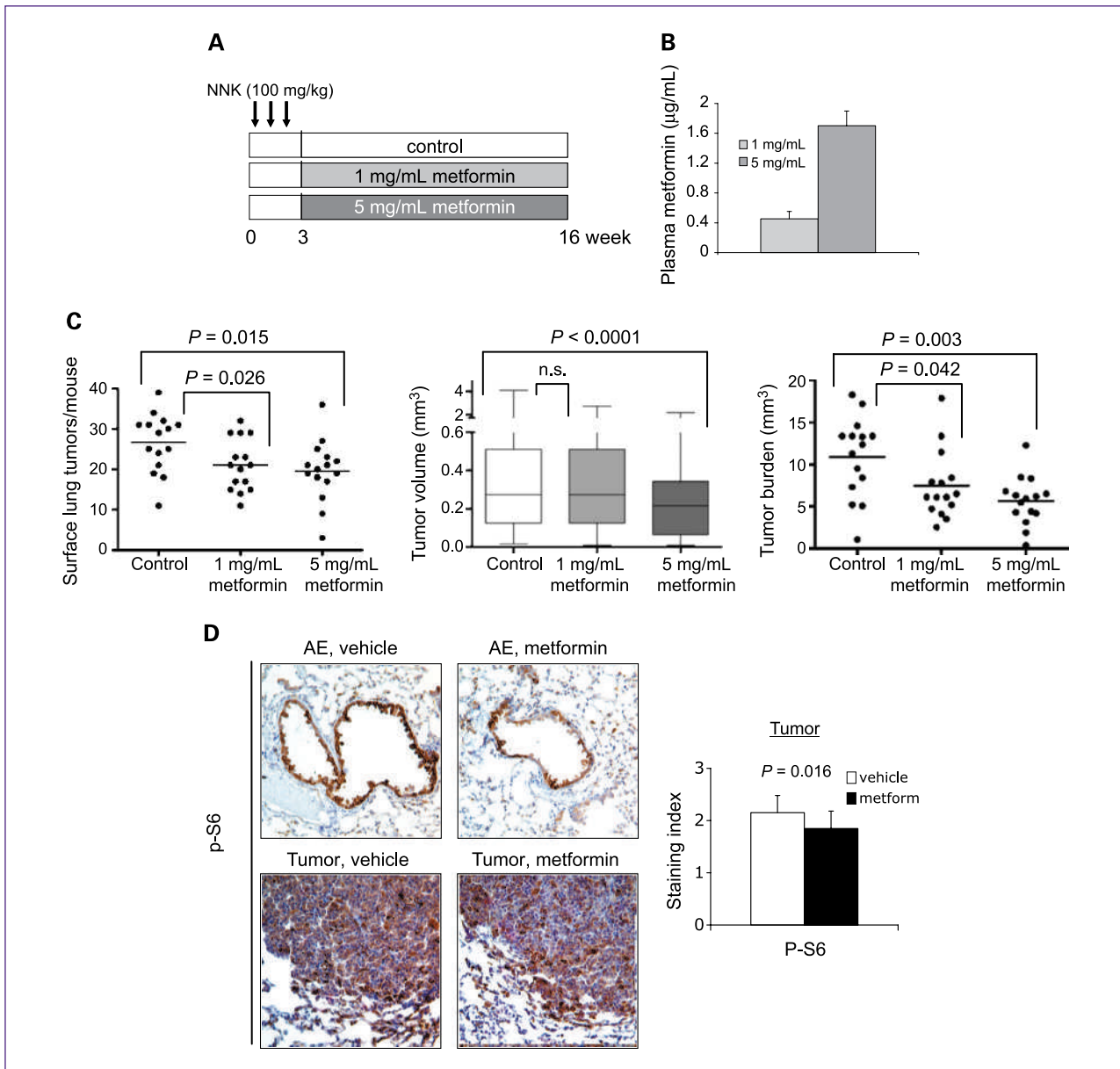


Fig. 1. Oral administration of metformin prevents NNK-induced lung tumorigenesis in A/J mice and is associated with modest inhibition of the mTOR pathway. **A**, schema for the prevention study with oral metformin in a tobacco carcinogen-driven mouse model of lung tumorigenesis. **B**, mean plasma levels of metformin in A/J mice treated with 1 or 5 mg/mL metformin orally were determined using liquid chromatography-tandem mass spectrometry. **C**, tumor multiplicity (left), tumor volume (middle), and tumor burden (right) were assessed after 13 wk of oral administration of 1 or 5 mg/mL metformin in A/J mice. For the dot plots, each point represents a mouse and the lines represent the median values. For the box and whisker graph, boxes indicate interquartile range, lines indicate median, and whiskers indicate minimal and maximal values. n.s., not significant. **D**, left, representative images of immunohistochemical analysis for p-S6 in airway epithelium (AE) and lung tumors from control mice and from mice that received oral metformin; right, quantification of immunohistochemical analysis. Columns, mean staining indices; bars, SD. Immunohistochemical analysis was done on five mice per treatment group.

Intraperitoneal administration of metformin produces higher plasma levels of metformin that cause tissue-specific modulation of the AMPK and mTOR pathways

Because oral metformin only modestly affected the mTOR pathway in lung tissue, we investigated if another route of administration with metformin might be more effective in modulating this pathway in mice. A short-term

biomarker study was done using intraperitoneal injections of metformin because we hypothesized that peak plasma concentrations would be higher than that achieved with oral administration. Pharmacokinetic analysis showed that plasma levels of metformin 2 hours after the last injection were higher (4.0 $\mu\text{g/mL}$) than with either oral dosing schedule. Immunoblotting analysis of lysates of liver

tissue harvested from mice treated with intraperitoneal metformin showed that it activated hepatic AMPK and increased the phosphorylation of a substrate of AMPK, acetyl-CoA carboxylase (Fig. 2A). Additionally, this dosing schedule with metformin inhibited the mTOR pathway in liver tissue, as shown by a trend toward decreased phosphorylation of both S6 and 4E-BP1, although some variability in these markers between mice was observed. Conversely, short-term oral administration of 5 mg/mL metformin did not activate AMPK or inhibit the mTOR pathway in liver tissue (Fig. 2B). This suggests that higher plasma metformin concentrations are better able to activate AMPK and inhibit the mTOR pathway.

Although intraperitoneal administration of metformin activated hepatic AMPK, it did not activate AMPK in lung tissue (Fig. 2C). Nonetheless, metformin still inhibited the mTOR pathway in lung tissue. Akt and ERK phosphorylation were also inhibited, suggesting that an upstream receptor

tyrosine kinase might be inhibited by metformin. Clinical studies have shown that metformin treatment is associated with decreases in plasma levels of insulin and IGF-I in both diabetic and nondiabetic patients (22, 23). Immunoblotting analysis using an antibody specific for the phosphorylated, or active forms, of the IGF-IR and IR showed that metformin decreased their activation in lung tissue. These results could suggest that intraperitoneal administration of metformin inhibits the mTOR pathway in lung tissue independently of AMPK by decreasing the response to insulin or IGF-I.

The fact that metformin activates AMPK in liver but not in lung tissue indicates a tissue-specific response to metformin, which could be related to differences in the level of expression of OCT1. This receptor is required for tissue uptake of metformin in mice, and polymorphisms in the gene that encodes OCT1 are associated with decreased response to metformin in diabetic patients (19). Quantitative

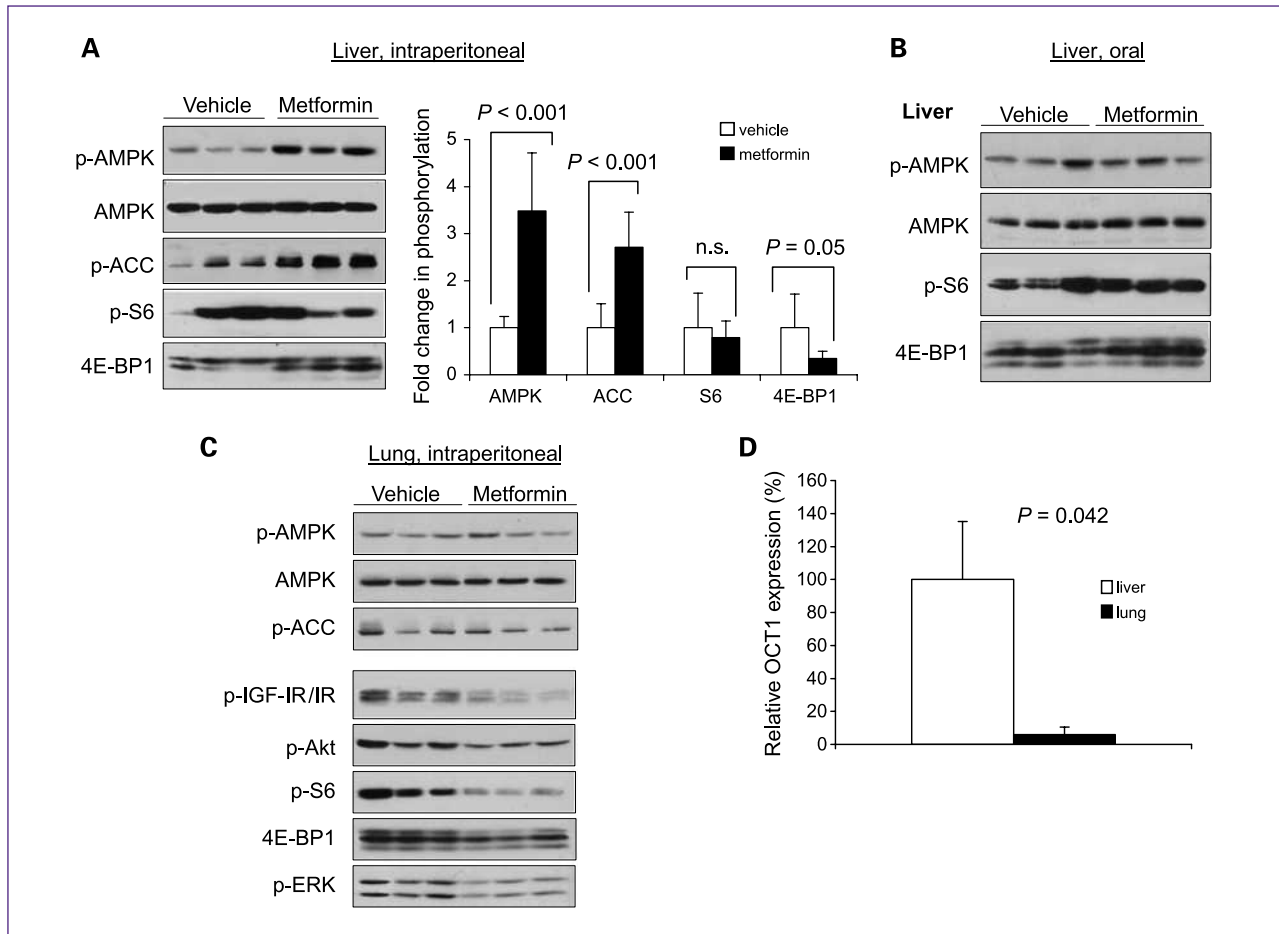
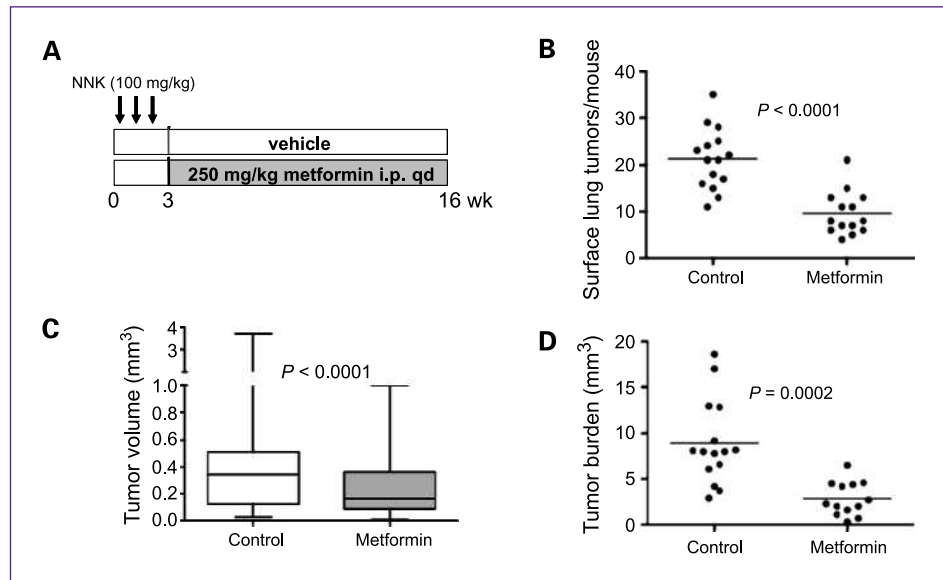


Fig. 2. Intraperitoneal administration of metformin causes tissue-specific modulation of the AMPK and mTOR pathways in A/J mice. A to C, immunoblotting analysis of components of the AMPK pathway and pathways downstream of IGF-IR/IR that converge on mTOR in liver (A and B) and lung (C) tissues. ACC, acetyl-CoA carboxylase. Tissues were harvested from mice treated short-term with intraperitoneal injections of 250 mg/kg metformin (daily \times 3) in two independent studies; representative blots from these studies are shown. Densitometry was done on immunoblots of liver lysates from both studies, which is presented in A (right). B, liver tissue was also harvested from mice treated with 5 mg/mL metformin orally for 7 d for comparison. D, OCT1 expression in liver and lung tissues from A/J mice was assessed by RT-PCR.

Fig. 3. Administration of metformin by intraperitoneal injection prevents NNK-induced lung tumorigenesis. A, schema for the prevention study with metformin administered by intraperitoneal injection in a tobacco carcinogen-driven mouse model of lung tumorigenesis. Tumor multiplicity (B), tumor volume (C), and tumor burden (D) were assessed after 13 wk of administration of 250 mg/kg metformin daily by intraperitoneal injection in A/J mice. For the dot plots in B and D, each point represents a mouse and the lines represent the median values. For the box and whisker graph shown in C, boxes indicate interquartile range, lines indicate median, and whiskers indicate minimal and maximal values.



RT-PCR analysis of liver and lung tissues harvested from A/J mice showed that OCT1 expression was 17-fold higher in liver tissue (Fig. 2D), suggesting that the inability of metformin to activate AMPK in lung tissue was due to diminished uptake.

Intraperitoneal administration of metformin is more effective than oral administration in preventing NNK-induced lung tumorigenesis in A/J mice

Because intraperitoneal administration of metformin significantly inhibited the mTOR pathway in lung tissue, we investigated if this dosing schedule with metformin was more effective in preventing NNK-induced lung tumorigenesis in A/J mice. Mice were fed the tumorigenic AIN-93G/M diets and treated with daily intraperitoneal injections of saline or metformin, beginning 1 week after NNK administration (Fig. 3A). This dosing schedule with metformin was surprisingly well tolerated and did not significantly affect the weight of mice throughout the course of the study (data not shown). Metformin was highly effective. Tumor multiplicity decreased by 66%, average tumor volume decreased by 50%, and overall tumor burden decreased by 72% (Fig. 3B-D). Phosphorylation of S6 in tumors decreased by 40% (Fig. 4A). Thus, intraperitoneal metformin was more effective in preventing NNK-induced lung tumorigenesis and inhibiting mTOR than oral metformin. However, consistent with the results of our short-term biomarker studies, this occurred in the absence of AMPK activation in lung tumors (Supplementary Fig. S1).

Previous studies have shown that metformin inhibits cancer cell proliferation *in vitro* by a mechanism that is dependent on inhibition of mTOR-induced protein translation (24). To investigate if metformin inhibits tumor cell proliferation *in vivo*, immunohistochemical analysis was done on lung tissues using an antibody specific for Ki-67, a marker of cellular proliferation (Fig. 4B). We ob-

served that inhibition of NNK-induced lung tumorigenesis by metformin was associated with a 67% decrease in tumor cell proliferation. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis showed that metformin did not induce apoptosis in tumor cells (data not shown). Collectively, these data show that prevention of NNK-induced lung tumorigenesis by metformin is associated with decreased tumor cell proliferation, but not increased apoptosis.

The ability of metformin to prevent NNK-induced lung tumorigenesis could also be due to the effects on the tumor microenvironment. We previously showed that NNK increases lung- and tumor-associated Foxp3⁺ Treg in A/J mice, which are required for K-ras-mediated lung tumorigenesis, and that rapamycin prevented the induction of lung-associated Foxp3⁺ cells by NNK (5). Because metformin also inhibits the mTOR pathway in NNK-induced lung tumors in A/J mice, we investigated if treatment with metformin was associated with decreases in lung- and tumor-associated Treg. Immunohistochemical analysis using antibodies specific for Foxp3, a marker of Treg, and CD3, a pan-T cell marker, showed that metformin treatment decreased lung-associated Treg by 65% and decreased tumor-associated Treg by 50% (Fig. 4C). Because we have previously shown that Foxp3⁺ Treg are required for K-ras-mediated lung tumorigenesis (5), decreases in lung- and tumor-associated Treg might contribute to the ability of metformin to prevent NNK-induced lung tumorigenesis in A/J mice.

Metformin is a well-tolerated drug that prevents fatty liver disease in A/J mice fed the tumorigenic diet AIN-93G/M

Because there were no published studies that showed the safety of daily intraperitoneal injections of metformin over many weeks, we harvested livers from mice at the end of the tumorigenesis study to assess hepatotoxicity. Examination

of H&E-stained sections showed that livers from the control group contained a large number of vacuoles that stained with Oil Red O (Fig. 5A). These lipid-laden vacuoles were substantially decreased both in size and number in livers from mice treated with metformin (Fig. 5B). The presence of vacuoles was most likely due to diet and not NNK because we did not observe liver vacuolization in mice that were exposed to NNK but were fed the cereal diet NIH-07 (Supplementary Fig. S2). Interestingly, the AIN-93G and AIN-93M diets are reformulations of the AIN-76A diet, which has been previously reported to cause fatty liver disease in rats (25). These results suggest that the tumorigenic AIN-93G and AIN-93M diets induce fatty liver disease in A/J mice, which is prevented by treatment with metformin. To confirm that diabetes was not induced by the AIN-93G/M diet (26), glucose and insulin levels were compared in mice fed either AIN-93G/M or NIH-07 diets. No differences in blood glucose (Fig. 5C) or insulin levels (data

not shown) were observed. This shows that metformin prevented tobacco carcinogen-induced tumor growth in a nondiabetic mouse model of lung tumorigenesis.

Inhibition of the mTOR pathway in tumors is associated with decreases in levels of circulating IGF-I and insulin

Because we showed that metformin decreased phosphorylation of IGF-IR and/or IR in lung tissue, and metformin is known to decrease circulating levels of these hormones in patients, plasma levels of insulin and IGF-I were assessed at the end of the tumorigenesis studies. Oral and intraperitoneal administration of metformin decreased insulin by ~20% and 35%, respectively (Fig. 6A). Additionally, oral metformin at either 1 or 5 mg/mL decreased the levels of circulating IGF-I by ~20% (Fig. 6B, left). Intraperitoneal injection of metformin did not decrease IGF-I levels, but the levels of IGF-I in mice injected with saline were

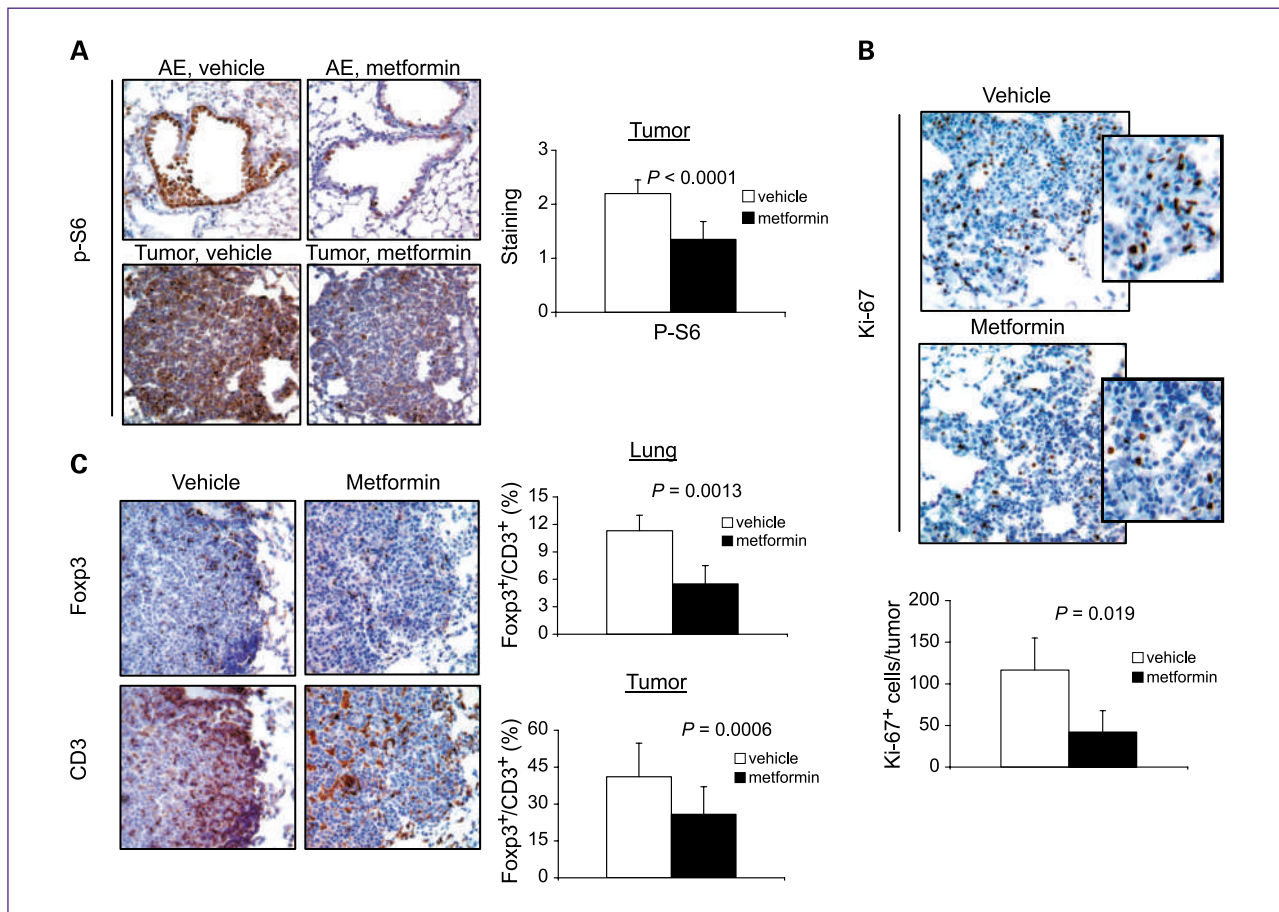


Fig. 4. Intraperitoneal administration of metformin inhibits the mTOR pathway in lung tissue, decreases tumor cell proliferation, and decreases lung- and tumor-associated Foxp3⁺ Treg. A, left, representative images of immunohistochemical analysis for p-S6 in airway epithelium (AE) and lung tumors from vehicle-treated mice and mice that received intraperitoneal metformin; right, quantification of immunohistochemical analysis. Columns, mean staining indices; bars, SD. B, top, representative images of immunohistochemical analysis for Ki-67, a marker of cellular proliferation, in lung tumors from vehicle- and metformin-treated mice; bottom, quantification of immunohistochemical analysis. Columns, mean number of Ki-67⁺ cells per tumor; bars, SD. C, left, representative images of immunohistochemical analysis for tumor-associated Foxp3⁺ and CD3⁺ cells in A/J mice treated with vehicle or intraperitoneal metformin; right, quantification of immunohistochemical analysis for lung- and tumor-associated percent Foxp3⁺/CD3⁺ cells (top and bottom, respectively). For A to C, immunohistochemical analysis was done on five mice per treatment group.

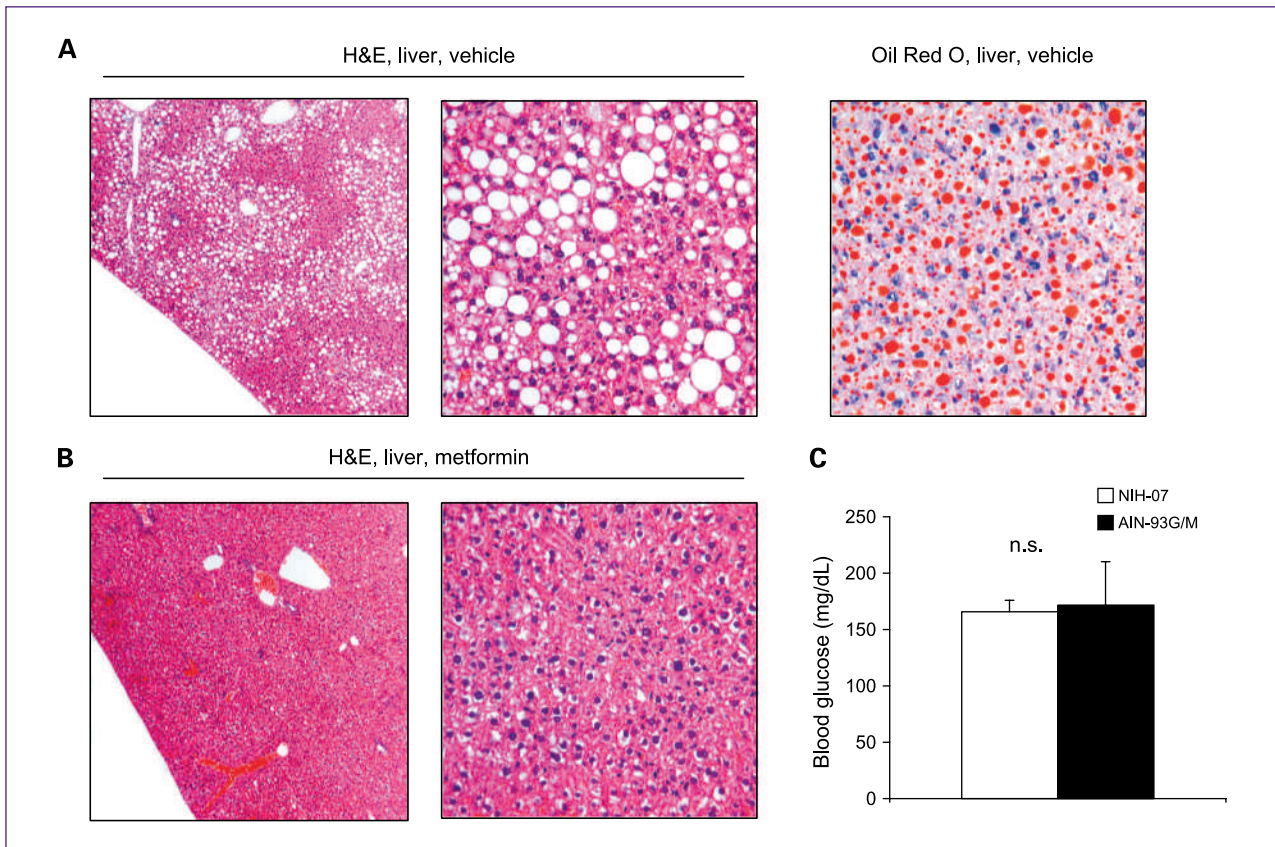


Fig. 5. The tumorigenic diet AIN-93G/M causes fatty liver disease in A/J mice, which is prevented by metformin, but does not cause hyperglycemia. A, H&E (left) or Oil Red O (right) staining of liver sections from A/J mice fed AIN-93G/M and treated with vehicle. B, H&E staining of liver sections from A/J mice fed AIN-93G/M and treated with 250 mg/kg metformin intraperitoneal daily for 13 wk. For A and B, images were taken at $\times 10$ and $\times 40$ magnifications. C, blood glucose levels in A/J mice that were fed the cereal diet NIH-07 or the tumorigenic diet AIN-93G/M ($n = 5$ mice per group). Columns, mean; bars, SD.

significantly lower than those in mice that were not injected (Fig. 6B, middle and right), suggesting that stress associated with daily injections could have obscured an inhibitory effect of intraperitoneal metformin on the circulating levels of IGF-I. These results support the hypothesis that metformin inhibits the mTOR pathway in normal lung epithelium and in lung tumors by decreasing circulating levels of IGF-I or insulin, which contributes to its ability to prevent NNK-induced lung tumorigenesis.

To investigate the responsiveness of lung and liver tissues to exogenously administered IGF-I or insulin, IGF-I or insulin was injected into the peritoneal cavity and tissues were processed for immunoblotting analysis. IGF-I rapidly activated the IGF-IR/Akt/mTOR pathway in lung tissue (Fig. 6C). It also increased phosphorylation of IGF-IR and Akt in liver tissue but did not affect the phosphorylation of S6. The tissue-specific response to insulin was opposite that of IGF-I, in that insulin had no effect on the mTOR pathway in lung tissue, but increased the phosphorylation of the IR, Akt, and S6 in liver tissue. These data suggest that lung tissue is more responsive to IGF-I and that liver tissue is more responsive to insulin. Given that metformin decreased the levels of IGF-I and

insulin, prevention of lung tumorigenesis by metformin might be related to multiple mechanisms in different organs.

Discussion

A majority of lung cancer cases are associated with tobacco use (85-90%), and more than 90 million current or former smokers in the United States are at permanent increased risk of developing lung cancer (27). Thus, there is a great need for the development of new chemotherapeutic strategies for the prevention of this disease. Our studies show that metformin is effective in preventing tumor growth in a mouse model of tobacco carcinogen-induced lung tumorigenesis. Oral administration of 1 or 5 mg/mL metformin decreased lung tumor burden in mice by 38% and 53%, respectively. The steady-state levels of metformin in mice given 5 mg/mL are similar to those in diabetic patients using metformin, suggesting the possibility that clinical prevention of lung cancer could be achieved with standard oral dosing. Despite this efficacy of oral metformin, intraperitoneal administration of metformin was more effective than oral administration and decreased

tumor burden by 72%. Because plasma levels of metformin were higher using this route of administration, these results could support the development of more potent analogues of metformin such as phenformin. Phenformin is more effective at decreasing hyperglycemia in diabetic patients, and preclinical studies show that phenformin is a more potent activator of AMPK and activates AMPK in a broader range of tissues than metformin (21). However, phenformin treatment is associated with lactic acidosis in patients, which could limit its clinical potential as a chemopreventive agent (28).

Inhibition of tumorigenesis by metformin could be related to mTOR inhibition within tumors that is an indirect effect of diminished circulating levels of growth factors such as insulin and IGF-I. Inhibition of mTOR correlated with decreased tumor cell proliferation in A/J mice, which is consistent with *in vitro* studies that show that metformin is a cytostatic agent that decreases cancer cell proliferation

by inhibiting mTOR-dependent protein translation (24). Metformin inhibited mTOR in lung tissue independently of AMPK activation, which suggests that lung tissue does not respond directly to metformin due to insufficient uptake. Consistent with this, lung tissues from A/J mice express 17-fold less OCT1 than liver tissues, which did show AMPK activation after metformin administration. Inhibition of the mTOR pathway by metformin was associated with decreased phosphorylation of IGF-IR/IR and decreased levels of circulating IGF-I and insulin, suggesting that they might contribute to tumorigenesis in this model. In agreement with this, a recent study using mice genetically engineered to overexpress IGF-I in lung tissues showed that IGF-I enhances NNK-induced lung tumorigenesis in FVB mice (29). These data suggest that the prevention of lung tumorigenesis by metformin could be due to effects on other tissues that decrease the circulating levels of growth factors.

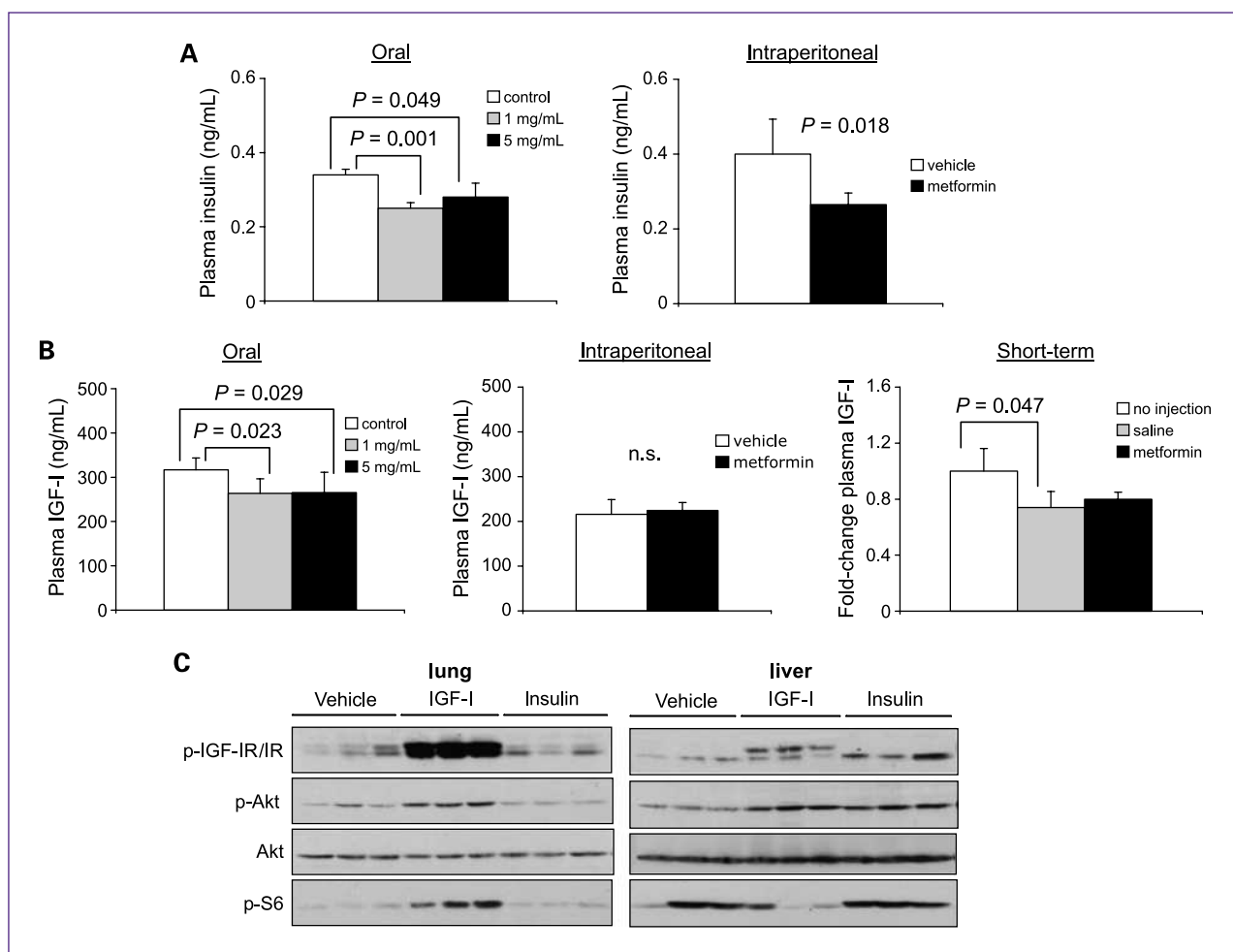


Fig. 6. Metformin-induced inhibition of the mTOR pathway in lung tissue is associated with decreases in circulating levels of IGF-I and insulin. A and B, plasma levels of insulin (A) or IGF-I (B) were assessed by ELISA in A/J mice treated with oral or i.p. administration of metformin at the end of the tumorigenesis studies. Columns, mean; bars, SD. This analysis was done on five mice per treatment group. Plasma IGF-I levels were also assessed in mice that were not injected or given intraperitoneal injections of saline or 250 mg/kg metformin daily \times 3 (B, right). C, immunoblotting analysis of the IGF-IR/IR/Akt/mTOR pathway in liver and lung tissues harvested from A/J mice 0.5 h after administration of 0.5 mg/kg IGF-I or 0.75 units of insulin.

This is the first study to show the safety and efficacy of metformin in a tobacco carcinogen-driven mouse model of lung tumorigenesis, but other studies have investigated the ability of metformin to prevent tumor growth *in vivo*. For example, studies performed using multiple xenograft models have shown that treatment with metformin modestly inhibited tumor growth (30–32). The modest effect of metformin observed in some of these xenograft models could be due to the use of immunocompromised mice or the fact that the subcutaneous compartment where tumors are placed is not analogous to the microenvironment within organs. A role for the microenvironment in the response to metformin is supported by the fact that metformin decreased lung- and tumor-associated Treg, which is a requirement for K-ras-induced lung tumorigenesis originally described in studies using rapamycin. In a syngeneic orthotopic model of lung cancer, LLC1, investigators showed that metformin effectively inhibited tumor growth, but only in mice that were fed a high-calorie diet (33). However, the role of the mTOR pathway in promoting tumor growth in this model is not clear, and inhibition of the mTOR pathway in tumors was not assessed. Metformin was also tested in a Pten^{+/-}Lkb1^{fl/+} mouse model (21). Pten and LKB1 are tumor suppressors that negatively regulate mTOR, and tissues from these mice were characterized by activation of the mTOR pathway. However, administration of metformin to Pten^{+/-}Lkb1^{fl/+} mice only increased tumor latency by 1 month and did not affect tumor incidence or morphology. Although this study used a similar oral dosing schedule, they did not observe decreases in serum levels of insulin. This could be related to loss of LKB1 and/or Pten in different tissues such as liver. Collectively, these studies suggest that indirect effects of metformin on the tumor microenvironment and on circulating levels of growth factors could be important in the prevention of tumorigenesis.

Metformin is a promising candidate for lung cancer chemoprevention. Clinical studies have shown that long-term treatment with metformin is associated with few adverse effects in diabetic, as well as nondiabetic, patient populations (7, 34). Similarly, the dosing schedules with metformin used in our studies seemed to be well tolerated and did not cause weight loss or hepatotoxicity. Metformin also prevented diet-induced fatty liver disease in A/J mice. This effect of metformin has been previously shown in mouse models of hepato steatosis (35), as well as in patients with nonalcoholic fatty liver disease (reviewed in ref. 36). Although the tumorigenic diets used in our studies caused lipid accumulation in the liver, they did not induce hyperglycemia or hyperinsulinemia. This shows that metformin was effective in preventing tobacco carcinogen-induced tumor growth in a nondiabetic mouse model of lung tumorigenesis. An additional advantage of metformin as a chemopreventive agent for lung cancer is that it inhibits tumor formation independently of AMPK activation in lung tissue. Metformin activates AMPK by a mechanism that is dependent on its upstream kinase, the tumor suppressor LKB1 (37). Somatic mutations in LKB1 are an early and frequent event in lung cancer and are associated with smoking (38–40). Because the effects of metformin are independent of AMPK in lung tissue, the presence of LKB1 mutations would not preclude response to metformin. Taken together, the safety and efficacy of metformin provide strong rationale for a clinical prevention trial with metformin in heavy smokers who are at high risk of developing lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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