MECHANISM AND TISSUE SPECIFICITY OF NICOTINE-MEDIATED LUNG S-ADENOSYL METHIONINE REDUCTION
Camilo A. Moncada1, Allen Clarkson2, Oscar Perez-Leal1 and Salim Merali1
From Department of Biochemistry, Temple University School of Medicine, Philadelphia PA1 and Department of Medical Parasitology, New York University School of Medicine, New York, NY2

Address correspondence to: Salim Merali Ph.D., Department of Biochemistry, Temple University School of Medicine, 3307 North Broad Street, Philadelphia, PA 19140, Tel.: 215 707-9228; Fax: 215 707-4568; Email: smerali@temple.edu

We previously reported that chronic nicotine infusion blocks development of Pneumocystis pneumonia. This discovery developed from our work demonstrating the inability of this fungal pathogen to synthesize the critical metabolic intermediate S-adenosylmethionine and work by others showing nicotine to cause lung-specific reduction of S-adenosylmethionine in guinea pigs. We had found nicotine infusion to cause increased lung ornithine decarboxylase activity (rate-controlling enzyme of polyamine synthesis) and hypothesized that S-adenosylmethionine reduction is driven by upregulated polyamine biosynthesis. Here we report a critical test of our hypothesis; inhibition of ornithine decarboxylase blocks the effect of nicotine on lung S-adenosylmethionine. Further support is provided by metabolite analyses showing nicotine to cause a strong diversion of S-adenosylmethionine towards polyamine synthesis and away from methylation reactions; these shifts are reversed by inhibition of ornithine decarboxylase. Because the nicotine effect on Pneumocystis is so striking, we considered the possibility of tissue specificity. Using laser capture microdissection, we collected samples of lung alveolar regions (site of infection) and respiratory epithelium for controls. We found nicotine to cause increased ornithine decarboxylase protein in alveolar regions but not airway epithelium; we conclude that tissue specificity likely contributes to the effect of nicotine on Pneumocystis pneumonia. Earlier we reported that the full effect of nicotine requires 3 weeks of treatment and here we show recovery is symmetrical, also requiring 3 weeks after treatment cessation. Because this time frame is similar to pneumocyte turnover time, the shift in polyamine metabolism may occur as new pneumocytes are produced.
15X reduction in lung AdoMet with no change in plasma or liver AdoMet. When nicotine was evaluated in a rat model of PCP, it was strongly protective (6). Compared to controls infused with saline, rats infused with 475 μg nicotine R- (+) kg⁻¹ hr⁻¹ starting at the time of inoculation had 99.9% fewer Pneumocystis organisms in their lungs 21 days post inoculation.

Understanding the shifts in metabolic pathways that drive nicotine-induced lung AdoMet depletion is interesting from a biochemical point of view and important for understanding how nicotine blocks development of PCP. Furthermore, it is possible that some part of nicotine toxicity relates to decreased lung AdoMet. We began our investigation by applying proteomic and biochemical techniques and results led to the hypothesis that polyamine metabolism is involved (6). We found that nicotine causes no change in lung activity of the AdoMet synthesizing enzyme methionine adenosyl transferase, thus we concluded that AdoMet synthesis is likely not affected. Since the major use of AdoMet is for methylation reactions, we looked for changes in response to nicotine. Shifts in methylation rates are reflected by changes in the “methylation index,” the ratio of the byproduct of methyl donation, S-adenosyl homocysteine, to AdoMet (7). This index increases whenever there is a significant increase in the rate of AdoMet-mediated methylation reactions for nucleic acid or protein methylation, for methionine regeneration, for lipid synthesis or for any other methyl donation pathway or combinations of pathways. We found nicotine treatment not to affect the lung methylation index, an observation confirmed by data in Table 2 of this publication, thus we concluded that AdoMet depletion was not driven by increased methylation activity. Using 2-D gel separation of protein extracts from lungs of rats treated with nicotine and controls, densitometry of gel spots for quantitation of differentially expressed proteins and peptide mass fingerprint for identification of these proteins, we found ornithine decarboxylase (ODC) to be upregulated 14X. Subsequent enzymatic assay of lung homogenates showed a 20X increase. ODC is the regulating enzyme for polyamine synthesis, an AdoMet-consuming pathway. The first steps of polyamine synthesis are parallel decarboxylations of ornithine by ODC and AdoMet by AdoMetDC; the former produces putrescine and the latter decarboxylated S-adenosylmethionine (dcAdoMet). Spermidine is the result of an aminopropyl transfer from dcAdoMet onto one of the amino groups of putrescine. Spermine results from transfer of a second aminopropyl group onto the other amino group of the putrescine core of spermidine. This increase in ODC led us to consider upregulation of polyamine metabolism as the mechanism underlying nicotine-induced lung AdoMet depletion (6). However, analysis of the polyamine content of lungs showed only an approximate 2X increase in putrescine, the product of ODC, and no change in the other major polyamines, spermidine and spermine, results confirmed by data in Table 1. One interpretation is that, despite changes in ODC, polyamine synthesis does not increase significantly and is not responsible for AdoMet depletion. An alternative hypothesis is that polyamine synthesis does increase, but catabolism also increases resulting in increased AdoMet consumption with little change in lung polyamine concentrations; we found evidence of increased polyamine catabolism. The key polyamine catabolic enzyme spermine/spermidine acetyl transferase (SSAT) is increased 4.5X in the lungs of nicotine-treated rats and N-1-acetylspermidine, a product of that enzyme, is increased 11X. The 2X increase in putrescine without increases in spermidine and spermine is consistent with increased catabolism in that enhanced SSAT activity will accelerate spermidine/spermine degradation while declining AdoMet concentration will cause slower spermidine/spermine synthesis thus increasing the ratio of putrescine to spermidine/spermine. Although these data support the hypothesis that nicotine causes lung AdoMet depletion by stimulating polyamine metabolism, the evidence is indirect. Here we report data directly demonstrating that nicotine-induced rat lung AdoMet depletion treatment is driven by increased polyamine metabolism. We also report that AdoMet reduction in response to nicotine is not a general lung response but is specific for the alveolar region, the site of Pneumocystis infection.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Supplies** DFMO was from ILEX Oncology, (San Antonio, TX). Ultrapure AdoMet,
spermidine, spermine, putrescine and R-(+)
nicotine were from Sigma-Aldrich Chemical Co. (St. Louis, MO). AccQ.Flour reagent kits were from Waters Corp. (Milford, MA). Dexamethasone sodium phosphate, 10 mg ml\(^{-1}\) for injection, was from Elkins-Sinn, Inc. (Cherry Hill, NJ).

**Administration of Drugs** - Nicotine and DFMO were delivered to animals by infusion pumps (Advanced Neuromodulation Systems, Plano, TX) implanted subcutaneously in the supra-scapular region as previously described (8). The pumps had a chamber volume of 1.0 ml, a flow rate of 500 \(\mu\)l day\(^{-1}\), and a catheter to deliver drug to the peritoneum. Pump solutions were changed as follows. The needle of an “infusion set” was inserted per-cutaneously into the pump fill port and the pump was drained into an attached syringe to measure the volume remaining; this allowed delivery rate calculation and confirmation of the manufacturer’s reported delivery rate when implanted and filled with our drug solution. To flush and refill, a second infusion set was inserted and 3 ml of fresh solution was slowly injected into the pump and drained through the first infusion set. The draining infusion set was then removed and 1.0 ml of fresh solution was injected to refill the pump reservoir. The 1.0 ml reservoir required re-filling every other day, but because AdoMet degrades rapidly, solutions containing AdoMet were replaced daily. At the time the pumps were implanted, the animals weighed 160-170 g. Immunosuppression by dexamethasone without \(P.\) carinii inoculation typically causes a weight drop to 130-140 grams after 4 weeks. Nominal dose rates reported in the results were calculated at the beginning of the experiment based on a mean body weight of 160 g; however, due to weight loss resulting from dexamethasone treatment, the dose rate can increase by as much as 20% by the end of the treatment period.

**Immunosuppression of Animals** - Specific pathogen-free SD rats (Taconic Farms, Germantown, NY) were housed in a barrier colony and maintained on multiple antibiotics to avoid other opportunistic infections, as previously described (9). The rats were pretreated with a combination of trimethoprim and sulfamethoxazole for 21 days to reduce latent infections. After trimethoprim and sulfamethoxazole treatment, infusion pumps were implanted and delivered saline during the 7 days allowed for recovery before immunosuppression was begun by adding dexamethasone in the drinking water (1.5 mg l\(^{-1}\)) and drug delivery was begun by changing pump solutions. This protocol was used because earlier nicotine-lung AdoMet data were collected with immunosuppressed rats. To control for dexamethasone, we performed an experiment using four groups of four rats each that were treated by pump infusion of the following: saline only, saline plus dexamethasone, nicotine only, and nicotine plus dexamethasone. After 3 weeks treatment, the animals were sacrificed and lungs analyzed for AdoMet. The respective mg AdoMet (g lung\(^{-1}\)) were: 6.78 ± 1.2, 7.22 ± 0.55, 0.78 ± 0.44 and 0.67 ± 0.23. Thus nicotine treatment caused an 88.5% reduction in lung AdoMet in animals not treated with dexamethasone and a 91.7% reduction in animals treated with dexamethasone. Because these results are similar, we conclude that dexamethasone does not interfere with the action of nicotine on lung AdoMet, nor is it required.

**Analysis for AdoMet, AdoMet metabolites, and polyamines** - The AdoMet contents of lung, liver and plasma samples were measured by HPLC analysis using Waters AccQ.Flour derivitizing reagent as previously reported (1). For biological samples, the limit of detection is 0.5 nmoles and linearity extends to 5,000 nmoles. All samples were analyzed in triplicate and the coefficient of variation ranged from 5 to 17%, depending on the amount of AdoMet in the sample. The polyamines spermidine, spermine and putrescine and AdoMet metabolites methylthioadenosine (MTA) and dcAdoMet were analyzed by HPLC using Waters’ AccQ.Flour derivitizing reagent as previously reported (10). Polyamine and AdoMet metabolite pools are expressed as nmol polyamine (g lung\(^{-1}\)).

**Analysis for nicotine** - Measurement of R-(+)
nicotine in plasma was by our capillary zone electrophoresis method (6). A P/ACE MDQ system equipped with a photodiode array detector allowed electropherograms to be monitored at 257 and 205 nm. Sample preparation involved adding 20 \(\mu\)l of 10% perchloric acid to 80 \(\mu\)l of plasma to precipitate proteins which were removed by centrifugation at 5000 g for 10 min. Supernatants were stored for up to 7 days at –20 °C before analysis. Just prior to analysis, samples were diluted 1:1 with water. Separation utilized an
amine capillary kit (Beckman, Inc.; 50 mm ID x 60 cm total length, 50 cm to the detector window) and the following protocol: 20 psi rinse with kit “amine regenerator solution” (2 min), 20 psi rinse with 50 mM Tris pH 8.0 buffer (2 min), sample injection, and separation using 30 kV in reverse polarity mode at 25 °C (7 min.). Specificity was assured by demonstrating that nicotine was resolved from all other peaks in plasma. Instrument precision was monitored by making triplicate injections from a single pooled standard.

Laser Capture Microdissection (LCM) - Sections (6 μm) were cut from frozen lung tissue specimens embedded in OCT using a OTF/AS cryostat (Jencons; Brigeville, PA) and mounted on PEN membrane slides (Arcturus; Mountain View, CA). The sections were stained with hematoxylin to reveal lung architecture and areas of interest collected using a Veritas (Arcturus) LCM instrument equipped with Veritas image archiving software. The laser cutting mode was used to cut out and collect selected regions of sections. The cutting beam diameter was 3.0 μM and the laser intensity control was set at 4.46 mV. Typically, 10-25 laser pulses were required to cut out the selected area which was then captured on mounted films (Arcturus) according to manufacturer directions. Portions of the films holding captured areas were placed in microfuge tubes for further processing.

ODC activity - Proteins were extracted from the pooled LCM material collected in microfuge tubes by adding NKPD buffer (2.68 mM KCl, 1.47 mM KH2PO4, 51.1 mM Na2HPO4, 7.43 mM NaH2PO4, 62 mM NaCl, 1 mM EDTA, and 1.0 mM DTT), vortexing, sonicating for 20 min at 40 watts and 70% duty cycle (Heat System, Ultrasonics Inc., Plainview, NY, USA), and clarifying by centrifugation at 30,000 g for 30 min. A 2 μl aliquot was retained for protein assay (Nanodrop Technology, Wilmington, DC) and the balance immediately used for ODC activity measurements as previously described in (11).

AdoMet decarboxylase (AdoMetDC) activity - A lung homogenate was prepared by freezing ~500 mg of tissue in liquid nitrogen then grinding in a mortar and pestle. The resulting powder was immediately resuspended in 2 ml of buffer (150 mM KCl, 2 mM dithiothreitol, 25 mM HEPES, 5 mM MgSO4), sonicated for about 2 min at 40 watts with a 70% duty cycle, and clarified by centrifugation at 10,000 x g for 15 min. An aliquot of the collected supernatant was used for protein assay (BioRad Bradford assay) and the balance for AdoMetDC assay. Assay conditions were as described for ODC except for substitution of substrate. Detection of the dcAdoMet product was as described above.

RESULTS

Nicotine and DFMO were delivered by implanted infusion pumps as previously reported (6,8) and summarized in Experimental Procedures. After surgically implanting saline-filled infusion pumps in the suprascapular region of groups of 5 rats each, saline was delivered over a 7 day surgical recovery period. After recovery, the pump solution for Group 1 rats was replaced with one containing 10.5 mg of R- (+) nicotine tartrate ml⁻¹ in saline to deliver a dosage of ~475 μg nicotine kg⁻¹ hr⁻¹. For Group 2, the exchange solution contained 94.5 mg DFMO ml⁻¹ to deliver a dosage of ~12 mg DFMO kg⁻¹ hr⁻¹. For Group 3, the exchange solution contained the nicotine content of Group 1 plus the DFMO content of Group 2. Group 4 rats were controls and the exchange solution was fresh saline. After 21 days of treatment, the 4 groups were sacrificed and lungs collected. Infusion of nicotine at this rate produced a blood concentration of 14.3 ± 2.5 μM at 21 days. Appropriate homogenates were prepared from portions of each lung with other portions immediately frozen in OCT embedding medium for subsequent sectioning. Aliquots of lung homogenate were used for analyses of polyamines, AdoMet, AdoMet metabolites and AdoMetDC activity.

Data in Table 1 show the effects of nicotine, DFMO, and the combination of nicotine plus DFMO on rat lung polyamines. Compared to saline-treated controls (Group 4), lung polyamine concentrations of DFMO-treated animals (Group 2) show sharp declines: putrescine ~9X, spermidine ~14X and spermine ~3X. Nicotine-treated animals (Group 1) show a 1.8X increase in lung putrescine but spermidine and spermine are essentially unchanged, results similar to those we previously reported (6). For animals given both nicotine and DFMO (Group 3), changes in lung polyamines are similar to those given DFMO alone: respective decreases of ~9X, ~19X and
These data show that, with respect to polyamines, the effect of DFMO cancels the effect of nicotine. Data in Table 2 show the effects of nicotine, DFMO, and the combination of nicotine plus DFMO on rat lung AdoMet, AdoMetDC and AdoMet metabolites. Compared to controls (Group 4), nicotine treated animals (Group 1) show ~10X decrease in AdoMet, ~9X increase in AdoMetDC, ~3X increase in dcAdoMet, and ~3X increase in MTA, all changes reflecting increased AdoMet flux through the polyamine synthesis pathway with attendant increased AdoMet consumption. Co-treatment with DFMO ablated the effect of nicotine.

LCM was used to collect samples from specific lung areas for measurement of ODC activity. Frozen sections from lungs of Group 1 nicotine-treated rats and Group 4 control rats were mounted, fixed and stained with hematoxylin and eosin. An LCM instrument was used to identify, cut and capture multiple samples of respiratory epithelium and alveolar regions. Figure 1 shows lung sections before and after sample collection by LCM. Panel A1 is a section containing an airway, A2 shows the same section after the airway epithelium has been cut and removed, and A3 shows the captured sample. B1, B2 and B3 show the equivalent images for capture of an alveolar region. Approximately 20,000 laser cuts/captures were performed for each area, for both nicotine treated and control animals. The pooled extracts of captured airway epithelium areas of control group rats contained 1.4 μg protein and alveolar areas 1.2 μg. For nicotine-treated rats, the corresponding values were 1.4 and 0.99 μg, respectively. Table 3 presents ODC specific activities of the extracts and also total lung homogenate. For the controls, ODC specific activities of airway epithelium, alveolar regions, and lung homogenate are roughly similar and the increase in activity induced by nicotine is specific for alveolar regions. Figure 2 shows the recovery of lung AdoMet after cessation of nicotine treatment. Infusion pumps were implanted into a group of 15 rats and nicotine was delivered at the same rate as above for 21 days after which time the pump solutions were replaced with saline. At days 1, 3, 7, 14 and 17 post cessation of nicotine treatment, 3 randomly chosen rats were sacrificed and the lungs collected for analysis of AdoMet content. Recovery of AdoMet after cessation of nicotine treatment occurred over 3 weeks, the same period required for depletion upon initiation of nicotine treatment.

**DISCUSSION**

A critical test of the hypothesis that nicotine causes lung AdoMet depletion by stimulating polyamine metabolism was to determine whether blocking polyamine synthesis would block nicotine-induced AdoMet depletion. One approach would be to inhibit the AdoMet-consuming step of polyamine metabolism, decarboxylation of AdoMet by AdoMetDC. Although many inhibitors of AdoMetDC are known and one is available in adequate amounts for in vivo experiments, all have metabolic effects beyond inhibition of AdoMetDC and these off-target effects would necessarily limit confidence in conclusions. An alternative approach was to block AdoMet consumption indirectly by inhibiting the other initial step of de novo polyamine synthesis, decarboxylation of ornithine by ODC to produce putrescine; limiting putrescine limits aminopropyl acceptors thereby restricting dcAdoMet use and thus consumption of AdoMet to produce dcAdoMet. We chose to block putrescine production because it is only one step downstream from direct inhibition of AdoMetDC and because an ideal tool was available: DL-α-difluoromethylornithine (DFMO). In contrast to inhibitors of AdoMetDC, DFMO is extraordinarily specific and has no other metabolic effects. This specificity is due to DFMO being an enzyme activated suicide inhibitor that is metabolically inert until the carboxyl group is cleaved by ODC. Cleavage activates the α-difluoromethyl group which alkylates a residue within the active site, cysteine-360, causing irreversible inhibition. A possible complication was that DFMO treatment has been found to cause a >10X upregulation in AdoMetDC and a >500X increase in dcAdoMet (12), effects that might have stimulated AdoMet consumption independent of nicotine thereby masking any reduction in nicotine-induced AdoMet consumption. However, not all cells respond similarly (13) and Table 2 data show that DFMO causes only moderate upregulation of rat lung AdoMetDC and accumulation of dcAdoMet: 2.0X and 21X increases in AdoMetDC and dcAdoMet, respectively. Nicotine treatment produces a greater increase in AdoMetDC, 8.5X,
but a smaller increase in dAdoMet, 2.6X, a pattern consistent with our hypothesis.

Table 1 data show that DFMO-treated animals have severe reductions in all three polyamines compared to controls, thereby demonstrating dosage suitability. The declines in putrescine and spermidine are greater than spermine suggesting that, despite blockage of de novo polyamine synthesis, some AdoMet consumption continues for spermine synthesis from pre-existing putrescine and spermidine, lower polyamines catabolically produced from spermine, and polyamines obtained from the diet. This is supported by Table 2 data that show the continuing presence of MTA in lung homogenates of DFMO-treated animals. In stark contrast to the 10X reduction in lung AdoMet of nicotine-treated animals compared to controls, the lung AdoMet content of animals treated with both nicotine and DFMO is essentially the same as animals treated with DFMO alone; i.e., DFMO reverses the effect of nicotine on lung AdoMet as predicted. Similarly, Table 1 shows the effects on polyamines are essentially the same for animals given DFMO treatment alone and for animals given both nicotine and DFMO. Table 2 shows that DFMO alone, or in combination with nicotine, causes a large increase in dAdoMet as expected since consumption of this intermediate is blocked by inhibition of putrescine production. Thus our hypothesis passed the critical test.

Other data in Table 2 provide additional support. Not only is lung AdoMet reduced by nicotine treatment, but also AdoMet catabolites dAdoMet and MTA are increased 2.6X and 2.7X, respectively. Since such catabolites can only be the result of AdoMet utilization for polyamine synthesis, these increases indicate increased flux in the polyamine pathway, as predicted. This shift is especially notable since it occurs in spite of severe AdoMet depletion and therefore indicates a strong diversion of AdoMet to polyamine metabolism. Although the 0.65 methylation index of Group 1 is similar to the 0.70 index of Group 4 controls, the absolute concentration of lung S-adenosyl homocysteine declines 10X in nicotine-treated animals compared to controls; this result also points away from increased activity in methylation pathways being involved in lung AdoMet depletion. Although it might seem that additional supporting data could be obtained by using DFMO to reverse the ability of nicotine to suppress PCP, this is not possible because DFMO itself is highly active against PCP both in the rat model (14) and clinically (15-19). Due to the very rapid response of this pathogenic fungus to manipulation of polyamine metabolism, DFMO is particularly effective when steadily infused (11,20).

There is precedence for increased polyamine flux causing cellular AdoMet reduction. When cells of the LNCaP prostate tumor line were genetically manipulated to increase SSAT mRNA 10X leading to a 20X increase in SSAT activity, the cells compensated for increased polyamine catabolism by increasing polyamine synthesis and the polyamine content of the cells did not decline (21). ODC activity increased 10X and AdoMetDC 8X. This increased polyamine flux required increased consumption of AdoMet and cell AdoMet content declined. With respect to the increase in putrescine and lack of change in spermidine and spermine content, the effects of nicotine on rat lungs and SSAT over-expression in LNCaP prostate tumor line cells are similar. Other effects, however, are dissimilar. The 20X SSAT activity increase in the cell line is greater than the 4.4X increase in lungs of nicotine-treated rats and the >1000X increase in the SSAT product N-1-acetylspermidine in the cell line is far greater than the 11X in the lungs. These data suggest a greater polyamine flux increase in the cell line than in the lung, yet AdoMet declined only 2X in the cell line overexpressing SSAT, far less than in lungs of rats treated with nicotine. It is possible that, despite the greater shift in enzyme activities, polyamine flux changes less in the cell line than in the lungs, but a more plausible explanation is that the cell line is better able to compensate for increased AdoMet consumption by increasing AdoMet production. This possibility brings up an interesting point. Increases in ODC and SSAT are common responses to stress, including exposure to nicotine (22,23), but a nicotine induced decline in AdoMet has never been described except for lungs. Therefore, the selective effect of nicotine on lung AdoMet may not depend so much on a selective increase in lung polyamine flux, but on an inability of lung cells to increase the AdoMet supply to compensate for increased consumption. Perhaps methionine needed for AdoMet synthesis is limited in lungs due to competing needs for 1-
carbon intermediates for lipid synthesis or perhaps lungs are unable to increase AdoMet synthesis for another reason. These questions cannot be answered with available data, but experimental approaches are feasible.

Even considering the remarkable degree of lung AdoMet depletion, the ability of nicotine to prevent PCP development in a rat model is extraordinary. We considered the possibility that AdoMet depletion may be especially marked in the cells *Pneumocystis* uses for attachment and nourishment: Type I pneumocytes of the lung parenchyma. To explore this, we used LCM to collect samples of lung parenchyma from sections of lungs taken from nicotine-treated and control rats; samples of airway epithelium were collected for the control (see Figure 3 for examples). Nicotine caused an 18X increase in alveolar region ODC activity, but only a 10% increase in airway epithelium activity (Table 3). The increases in lung parenchyma and total lung homogenate are similar, reflecting the relative composition of the lung. Although these data show lung AdoMet depletion is not general, LCM resolution does not allow discrimination among various cell types present in the alveolar region: Type I pneumocytes, Type II pneumocytes, macrophages, fibroblasts, capillary endothelia and blood cells within the capillaries. One could speculate that Type I pneumocytes are the target because they are the most common cell type of the parenchyma and *Pneumocystis* adheres to them. However, since nicotine causes a 10X AdoMet reduction overall (6,14) and the parenchyma is not 90% Type I cells, this would mean that the baseline AdoMet in all other lung parenchyma cells would have to be very low if depletion of Type I cells results in 10X reduction of lung AdoMet. While data for AdoMet concentrations within lung regions is unavailable, broadening the speculation to include both Type I and II pneumocytes reduces this problem since these cells do comprise the bulk of the parenchyma. Although Type I and II cells have different morphologies, compositions and functions, one develops directly from the other so it is not unreasonable to speculate that they respond similarly to nicotine. This is another interesting question requiring further work for resolution.

A recent paper reported the effects of smoking and nicotine on related aspects of lung metabolism (24). But because the focus of that work was on asthma, the concentration was on airway epithelium, not alveolar regions. These authors found airway epithelium to show increases in expression of both ODC and arginase I, the enzyme that produces the ornithine substrate for ODC. Results were based on immunochemistry to detect protein and *in situ* hybridization to reveal corresponding mRNA. Quantification was by visual inspection of coded slides. They also studied the response of cultured cells to nicotine stimulation using quantitative PCR. These authors report a 2X increase in airway epithelium ODC protein and mRNA in sections and a 1.8X increase in mRNA in cultured cells. In contrast, we found only a 10% increase in ODC enzyme activity in airway epithelium. We cannot explain this difference, but note the different techniques used and the fact that their samples were from asthmatic patients, smokers and non-smokers, and cultured cells while our samples were from otherwise healthy laboratory animals, some treated with nicotine and some not. Besides those differences, they measured ODC protein and mRNA and we measured activity in these experiments. Since control of ODC activity involves many post-translational mechanisms in addition to control of expression, it is possible that both sets of results are correct and would be the same even if the samples were the same. This is a very new area of investigation and better explanations will likely arise with further work.

We previously showed rat lung AdoMet declines slowly upon initiation of nicotine treatment (6) and data here (Figure 2) show recovery upon withdrawal is also very slow. The curves are mirror images with each requiring about 3 weeks for completion. Since pneumocytes are replaced over a similar time period (25), the notion arises that the presence of nicotine may cause a change in the phenotype of pneumocytes as they are produced in a manner reminiscent of enterocyte iron transport phenotype being dependent on iron stores at the time they are produced (26). However, other data show a rapid increase in lung ODC upon exposure to nicotine and cigarette smoke and therefore argue against this notion (24,27,28). It may be that nicotine causes relatively rapid changes in enzyme activities but these cause only a slightly greater rate of AdoMet consumption than production thus lung AdoMet...
declines slowly and recovers slowly upon removal of nicotine. This remains an open question.

The possibility of nicotine being used to treat PCP or to prophylax against this disease is another issue, one beyond the scope of this report. However, we do note that the 9.6 mg kg\(^{-1}\) day\(^{-1}\) dose of nicotine used to collect most of the data presented here produces a steady state plasma concentration of 2.32 \(\mu\)g ml\(^{-1}\), near a toxic level. But 1\% of this dose, or 96 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), produces a steady state plasma concentration of 300 nM or 4.9 ng ml\(^{-1}\) and, when given to immunosuppressed rats starting at the time of inoculation with \(P.\ carinii\), reduces the lung burden at 3 weeks by 85\% compared to saline-treated controls (6).

Further insight is offered by the results of a human study using well tolerated nicotine-containing gum or lozenges that are ordinarily used as aids for those trying to cease smoking or chewing tobacco. Lozenges and gum containing 4 mg of nicotine produced plasma nicotine concentrations rising over the first hour to approximately 10 ng ml\(^{-1}\), dropping to about 5 ng ml\(^{-1}\) at 3 hrs and dropping further to about 3 ng ml\(^{-1}\) at 6 hrs. Because these plasma concentrations exceed those that suppressed PCP by 85\% in rats, it is possible that a non-toxic nicotine dose could be clinically active against PCP. Furthermore, once the initial interaction between nicotine and lung cells that initiates increased polyamine cycling is better understood, it might be possible to identify analogues that retain this activity but are less toxic than nicotine.

In summary, data here demonstrate that nicotine-induced lung AdoMet depletion is tied to increased polyamine metabolism. This effect on AdoMet is not general, but is restricted to alveolar regions of the lung. We know that AdoMet depletion is a slow process and recovery is as well, but we don’t know if this is due to a nicotine-mediated phenotype change as pneumocytes are produced or to a small but persistent change in the net difference between AdoMet production and consumption. As far as is known, the effect of nicotine on cell AdoMet content is specific for lungs, but it is unknown whether this is because lungs respond to nicotine by increasing polyamine metabolism more strongly than other organs or whether this is a general cell response to nicotine that has a greater effect on lungs because they are less able to compensate by increasing AdoMet production.

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**FOOTNOTES**

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**FIGURE LEGENDS**

Figure 1. Laser Capture Microdissection. These images illustrate marking, cutting and capture of selected regions of lung sections. A1 and A2: Areas containing airway epithelium and alveoli, respectively, as marked by software for subsequent cut and capture. B1 and B2: Same sections after the marked areas were cut and removed. C1 and C2: Captured areas.

Figure 2. Recovery of lung AdoMet after cessation of nicotine treatment. Each point represents the mean value for lung homogenate AdoMet assays for three animals sacrificed at the indicated time post cessation of nicotine treatment.
Table 1 - Polyamines in response to treatment with nicotine, DL-difluoromethylornithine (DFMO), and nicotine plus DFMO:

| Group                      | Putrescine* | Spermidine* | Spermine*   |
|----------------------------|------------|-------------|-------------|
| 1 - Nicotine**             | 394 ± 66   | 813 ± 38    | 1522 ± 78   |
| (475 μg/kg/hr)             |            |             |             |
| 2 - DFMO**                 | 27 ± 6     | 59 ± 15     | 579 ± 122   |
| (12 mg kg⁻¹ hr⁻¹)          |            |             |             |
| 3 - Nicotine (475 μg kg⁻¹ hr⁻¹) + DFMO (12 mg kg⁻¹ hr⁻¹) | 24 ± 15   | 45 ± 21     | 622 ± 152   |
| 4 - Saline                 | 221 ± 45***| 844 ± 72*** | 1645 ± 112***|

* nmol (g wet tissue)⁻¹

** Nicotine and DFMO were dissolved in saline. Infusion pumps delivered a volume of 21 μl hr⁻¹.

*** Data for Group 2 were collected separately than for the other groups, but exactly the same protocols were used and a separate group of saline-treated controls was included. The putrescine, spermidine and spermine concentrations of Group 2 controls were 244 ± 38, 828 ± 66, and 1592 ± 98 nmol (g wet tissue)⁻¹, respectively. Since these are essentially the same as the Group 4 controls in this table and all comparative ratios are the essentially same regardless the set of controls used for calculation, we included Group 2 data in this table. However, the correct control data set was used to calculate changes induced by DFMO alone.
Table 2 - AdoMet and AdoMet metabolites in response to treatment with nicotine, DL-difluoromethylornithine (DFMO), and nicotine plus DFMO:

|                      | AdoMet* | AdoMetDC** | dcAdoMet* | MTA*  | AdoHcy* |
|----------------------|---------|------------|-----------|-------|---------|
| **Group 1 - Nicotine*** | 2.0 ± 0.4 | 6.12 ± 1.6 | 4.7 ± 1.8 | 8.8 ± 1.3 | 1.3 ± 0.4 |
| (475 μg/kg/hr)*      |         |            |           |       |         |
| **Group 2 - DFMO***   | 22 ± 2.3 | 1.5 ± 0.4  | 38 ± 6    | 3.5 ± 0.9 | 16.1 ± 1.2 |
| (12 mg kg\(^{-1}\) hr\(^{-1}\))* |         |            |           |       |         |
| **Group 3 - Nicotine** | 25 ± 4.2 | 1.3 ± 0.3  | 32 ± 5.2  | 2.7 ± 0.6 | 13.8 ± 3.9 |
| (475 μg kg\(^{-1}\) hr\(^{-1}\)) + DFMO (12 mg kg\(^{-1}\) hr\(^{-1}\)) |         |            |           |       |         |
| **Group 4 - Saline**  | 20 ± 3.3*** | 0.68 ± 0.2 | 1.8 ± 0.4*** | 3.3 ± 0.9*** | 14 ± 3.2*** |

* nmol (g wet tissue)\(^{-1}\)
** nmol dcAdoMet (mg protein)\(^{-1}\) h\(^{-1}\)
*** Nicotine and DFMO were dissolved in saline. Infusion pumps delivered a volume of 21 μl hr\(^{-1}\)
**** Data for Group 2 were collected separately than for the other groups, but exactly the same protocols were used and a separate group of saline-treated controls was included. Control group values for AdoMet, AdoMetDC, dcAdoMet, MTA and AdoHcy were 22 ± 2.8, 0.74 ± 0.5, 1.7 ± 0.3, 3.5 ± 1.1 and 12.1 ± 3.4 nmol (g wet tissue)\(^{-1}\), respectively. Since these are essentially the same regardless the set of controls used for calculation, we included Group 2 data in this table. However, the correct control data set was used to calculate changes induced by DFMO alone.
Table 3 - Ornithine decarboxylase activity in alveoli and airway epithelium regions collected from lung sections of Group 1 (nicotine-treated) Group 4 (saline-treated) animals by laser capture microdissection (n = 3).

| Sample                  | ODC activity (nmol putrescine (g tissue)$^{-1}$ hr$^{-1}$) |
|-------------------------|-----------------------------------------------------------|
|                         | Group 4 Saline-treated | Group 1 Nicotine-treated |
| Alveolar Region         | 4.7± 1.8               | 83 ± 12                  |
| Airway Epithelium      | 5.4 ± 2.1              | 6.1 ± 1.9                |
| Lung Homogenate        | 4.1± 1.1               | 78 ± 14                  |
Figure 1

Epithelial cells

Alveoli

A1

B1

C1

A2

B2

C2
Figure 2

Days post nicotine cessation
Mechanism and tissue specificity of nicotine-mediated lung S-adenosylmethionine reduction
Camilo Moncada, Allen B. Clarkson, Oscar Perez-Leal and Salim Merali

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