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Involvement of Semicarbazide-Sensitive Amine Oxidase-Mediated Deamination in Lipopolysaccharide-Induced Pulmonary Inflammation

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Semicarbazide-sensitive amine oxidase (SSAO) resides on the vascular endothelium and smooth muscle cell surface and is capable of deaminating short chain aliphatic amines and producing toxic aldehydes and hydrogen peroxide. The enzyme, also known as a vascular adhesion protein-1, is involved in the inflammation process. This intriguing protein with dual functions is increased in the serum of diabetic and heart failure patients. In the present study we assessed the involvement of SSAO in a lipopolysaccharide-induced pulmonary inflammation model using transgenic mice that overexpress human vascular adhesion protein-1. Overexpression of SSAO activity increased the formation of protein-formaldehyde deposits in tissues. Lysine residues of proteins were the primary targets for cross-linkage with formaldehyde derived from deamination of methylamine. Lipopolysaccharide-induced increases in inflammatory cells in the bronchoalveolar lavage (BAL) fluid were significantly higher in the transgenic than in the nontransgenic mice. BAL cell counts were also higher in the untreated transgenic than in nontransgenic mice. Blocking SSAO activity with a selective inhibitor significantly reduced the number of neutrophils as well as levels of macrophage inflammatory protein-1α, granulocyte colony-stimulating factor, tumor necrosis factor-α, and interleukin-6 in the BAL fluid. Inhalation of methylamine also increased BAL neutrophil counts. Together, these results suggest a role for SSAO-mediated deamination in pulmonary inflammation. ***(Am J Pathol 2006, 168:718–726; DOI: 10.2353/ajpath.2006.050970)***

Semicarbazide-sensitive amine oxidases (SSAO) are a group of enzymes that contain copper and quinone, and are sensitive to semicarbazide.[1] The enzyme is located on the outer cell surface of and associated to vesicles in vascular smooth muscle and endothelial cells as well as on or in adipocytes.[2] A soluble form also circulates in the blood.[3] Increased serum SSAO activities were found in patients with diabetic complications, vascular disorders,[4–6] and heart failure.[7] Increased serum SSAO has been considered a risk factor for vascular disorder in heart failure.[8] Interestingly, SSAO-mediated deamination of methylamine and aminoacetone leads to the production of toxic formaldehyde and methylglyoxal, respectively, as well as hydrogen peroxide and ammonium.[9,10] These toxic products may be responsible, at least in part, for protein cross-linkage, oxidative stress, and cytotoxicity associated with these pathological conditions.[1]

A protein called a vascular adhesion molecule (VAP-1) was independently discovered and was found to be identical to SSAO.[11] It is located on the endothelial surface and participates in the adhesive events between leukocytes and the vascular wall.[12] VAP-1 has been shown to affect leukocyte trafficking to sites of inflammation in mice, including acute inflammation in experimental peritonitis and air pouches as well as chronic inflammation associated with diabetes.[13] Interestingly, the enzymatic activity of SSAO (VAP-1) is required for granulocyte extravasation through the endothelium.[14] This transmigra-

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tion process exhibits a unique mechanism, namely, both the adhesive and catalytic functions are necessary for the physiological function.[14]

That the lung possesses relatively high SSAO activity in rodents and humans was observed 2 decades ago.[15,16] The lung SSAO is important in metabolism of amines.[17] Although the exact function of the lung in SSAO-mediated deamination is unknown, it was demonstrated that VAP-1 (SSAO) might be involved in perivascular recruitment of inflammatory cells during lung inflammation.[18] Overexpressing SSAO may result in abnormal structure of aortic elastic laminas and alteration of the elasticity of the blood vessels.[19] It has been demonstrated that SSAO-mediated deamination is vasoactive.[20] SSAO catalyzes the deamination of some volatile short-chain aliphatic amines, such as methylamine, and produces toxic aldehydes, which has been shown a primary target at the lysine residues of proteins.[21] Transgenic mice overexpressing human VAP-1 (SSAO) targeted to endothelial cells using a TIE-1 promoter were recently generated.[22] In the present study we report that the overexpression of SSAO activity in these transgenic mice is particularly pronounced in lung in comparison to other tissues. We have therefore used these mice to investigate the role of lung SSAO involved in pulmonary inflammation.

Materials and Methods

Materials

[^{14}C]-Methylamine hydrochloride (52 mCi/mmol) was purchased from New England Nuclear/Du Pont (Mississauga, ON, Canada). (E)-2-(4-fluorophenetyl)-fluoroallylamine, (MDL-72974A) was kindly provided by Marion-Merrell-Dow Inc. (Cincinnati, OH). Methylamine, *Escherichia coli* lipopolysaccharide (LPS) and fluorenylmethyl chloroformate (FMOC)-Cl was obtained from Sigma (St. Louis, MO), and Diff-Quick was from Dade Behring Inc. (Newark, DE). All other chemicals are of analytical grade.

Animals

Male CD-1 mice (20 to 25 g) were obtained from Charles River, St. Constant, QC, Canada. mTIEVAP-1 transgenic mice overexpressing human endothelial SSAO were created as previously described.[3] The mice were maintained on the FVB/n inbred genetic background. Homozygous and nontransgenic lines were derived from heterozygous intercrosses. The animal studies were in strict accordance with guidelines established by the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee. Rodents were housed in hanging wire cages with free access to food and water on a 12-hour light/dark cycle (lights on at 6 a.m.) at a temperature of 19 to 20°C.

LPS-Induced Acute Lung Inflammation and Bronchoalveolar Lavage (BAL) Cell Counts

Mice were anesthetized with halothane. They subsequently received 50 μl of LPS (2 μg/animal) through the nose with a micropipette. Such a dose is known to produce a maximal neutrophil accumulation in the alveolar space.[23] Control animals received vehicle only. Mice were killed in a CO_2 chamber 24 hours after LPS instillation. BAL was obtained using three washes of 1 ml of saline.[24] The first retrieved aliquot was centrifuged independently from the two last ones. The first cell-free aliquot of BAL fluid was kept at −70°C for biochemical analyses. Total cell counts were conducted from BAL cells from the three combined aliquots resuspended in phosphate-buffered saline (PBS) with a grid hematocytometer. Diff-Quick stain was also used for microscopic examination of the BAL cells.

SSAO Activity Assay

SSAO activity was assessed by a radio-enzymatic procedure using ^{14}C-labeled benzylamine as the substrate.[25] The SSAO enzyme preparations were incubated with clorgyline (10^{-6} mol/L) and (−)-deprenyl (10^{-6} mol/L) at 37°C for 20 minutes to ensure that any monoamine oxidase activity was completely blocked. Aliquots of the enzyme preparation were then incubated with 50 μl of [^{14}C]-benzylamine (4×10^{-4} mol/L, 1 μCi/ml) in a final volume of 200 μl in phosphate buffer (0.1 mol/L, pH 7.4) at 37°C for 30 minutes. Enzyme reaction was terminated by adding 250 μl of 2 mol/L citric acid. The oxidized products were extracted into 1 ml of toluene:ethyl acetate (1:1, v/v), of which 600 μl was transferred to a counting vial containing 10 ml of ACS scintillation cocktail (Amersham Radiolabeled Chemicals Inc., St. Louis, MO). Radioactivity was assessed in a LS-7500 liquid scintillation counter (Beckman, Fullerton, CA). Tissues were dissected and kept frozen at −70°C. The tissues were then homogenized with a Polytron homogenizer (Kinematica GMBH, Luzern, Switzerland) followed by brief sonication before determination of SSAO activity.

Urine Collection

Mice were placed in metabolic cages (Nalgene, Rochester, NY) for urine collection throughout a period of 20 hours. The urine-collecting vessels were positioned over Styrofoam containers filled with dry ice, which permitted the freezing of urine immediately after urination. The animals were allowed free access to tap water. During urine collection, food was withheld.

Analysis of Methylamine

Urinary methylamine was measured using a high performance liquid chromatography (HPLC)-fluorometric procedure as previously described.[26] The urine samples after purification using a small CG-50 Amberlite column were precolumn derivatized with *o*-phthaldialdehyde and

then applied to a Beckman Ultrasphere IP column of octadecyl-bonded spherical-5 μm silica particles, using a Shimadzu HPLC system (Sil-9A autoinjector) equipped with a precolumn derivatization program. The column was eluted with 65% methanol at a flow rate of 1.0 ml/minute. For quantitative assessment, a programmable fluorescence detector (HP1046A; Hewlett Packard) with excitation at 360 nm and emission at 445 nm was used. Isopropylamine was used as an internal standard.

Formaldehyde-Protein Adducts Derived from SSAO-Mediated Reactions

An HPLC/FMOC procedure was used for the detection of cross-linkage of lung proteins by formaldehyde derived from the deamination of methylamine.[21] Lung tissue slices (100 μm^2) were incubated with methylamine (10^{-3} mol/L in 0.02 mol/L phosphate buffer, pH 7.4) at 37°C for 4 hours. Then 1 ml of 10 mmol/L $NaCNBH_3$ was added, and the samples were mixed and further incubated at 37°C for 24 hours. Lung tissues from both nontransgenic and transgenic mice were used. In addition, a group of transgenic mice were treated with MDL-72974A (5 mg/kg) to block SSAO activities before performing this experiment. The reaction mixtures were then dialyzed extensively (Spectr/Por cellulose membrane, MWCO: 3.5 kd; Spectrum Laboratories, Rancho Dominguez, CA) with three changes of 0.2 mol/L phosphate buffer (pH 8.0). The formaldehyde-protein adducts were hydrolyzed in 6 N of HCl at 105°C for 24 hours.

Precolumn Derivatization of Amino Acids with FMOC-Cl

The hydrolyzed samples were neutralized with 10 mol/L NaOH. To each 100-μl sample, 50 μl of potassium borate buffer (0.8 mol/L, pH 10) was added and the solution was vigorously vortexed for 1 minute. One hundred μl of FMOC-Cl solution (10 mmol/L in dehydrated acetonitrile) was added and the solution was immediately vortexed for 1 minute. One ml of hexane was then added and the reaction mixture was vigorously shaken for 45 seconds, centrifuged, and the hexane phase containing excess reagent was discarded. The extraction was repeated twice. A 10-μl aliquot of acetic acid (20 v/v percent) was added, the tube was mixed, and the sample was applied to HPLC analysis.

Chromatography

The HPLC system consisted of a Shimadzu LC-10AD VP delivery system, a DGU-14A degasser, a SIL-10AD VP autoinjector (Man-Tech, Guelph, ON, Canada), and an integrator (Spectra-Physics, San Jose, CA). The separation was performed using a reverse-phase Ultrasphere LP analytical column (4.6 $\times$ 250 mm, 5 μm; Beckman, Toronto, ON, Canada). Elution was either isocratic with 0.05 mol/L sodium-acetate buffer (pH = 5.0, 43 v/v percent) in acetonitrile (flow rate, 1.4 ml/minute), or a ternary gradient system, in which solvent A was 20 mmol/L citric acid containing 5 mmol/L tetramethylammonium chloride adjusted to pH 2.85 with 20 mmol/L sodium acetate; solvent B was composed of 80% (v/v) 20 mmol/L sodium acetate solution containing 5 mmol/L tetramethylammonium (adjusted to pH 4.5 with concentrated phosphoric acid) and 20% (v/v) methanol; and solvent C was acetonitrile. The gradient program is according to Bank and colleagues.[27] The separation was performed at room temperature. Spectrophotometric detection was conducted using a Lambda-Max model 481 LC spectrophotometer (Waters; Millipore, Mississauga, ON, Canada) at a wavelength of 265 nm. Data represent the average of at least three analyses.

Distribution of Residual Radioactivity after Administration of [^{14}C]-Methylamine

The experimental procedure has been previously described.[23] Briefly, radioactively labeled methylamine (5 μCi, 100 nmol) was administered to the mice via tail intravenous injection. After 72 hours the mice were killed and different tissues were dissected. Aliquots of these tissues were homogenized in 0.05 mol/L phosphate buffer (pH 7.2) (1:3, w/v) and aliquots of the homogenates were transferred to counting vials containing 0.5 ml of Solvable and 10 ml ACS scintillation fluid (Amersham, Oakville, ON, Canada). Radioactivities were assessed by liquid scintillation spectrometry (LS-6500, Beckman).

Tumor Necrosis Factor (TNF)-α Analysis

TNF-α level in the BAL fluid was analyzed by enzyme-linked immunosorbent assay (ELISA). High-affinity 96-well microtiter plates (Maxisorb, Nunc) were coated with goat anti-TNF-α IgG. The plates were thoroughly washed and blocked bovine serum albumin. BAL samples were added into the plate and incubated at least 2 hours at room temperature. After washing, biotinylated goat anti-TNF-α IgG containing 100 ng/ml in PBS-TB was added. Thereafter, extravidin-horseradish peroxidase, diluted in 1:1000 in PBS-TB, was added. After washing, immunoreactivity was detected by adding tetramethylbenzidine dihydrochloride as peroxidase substrate and incubated for 30 minutes at room temperature. The reactions were stopped by the addition of H_2SO_4. The optical density was determined at 450 nm by using a plate spectrophotometric plate reader and data were analyzed by Softmax Pro software. Dose-response standard curves of TNF-α between 50 pg/ml and 4 ng/ml were obtained.

Cytokine Antibody Arrays

Cytokine levels in the BAL fluids were also analyzed by an antibody array procedure. A TranSignal mouse kit (MA-6412; Panomics Inc., Redwood City, CA) was used. The assay is based on the sandwich ELISA and simultaneously analyzes 16 cytokines (please see details in Figure 7) in the BAL fluid used for the assay. The method

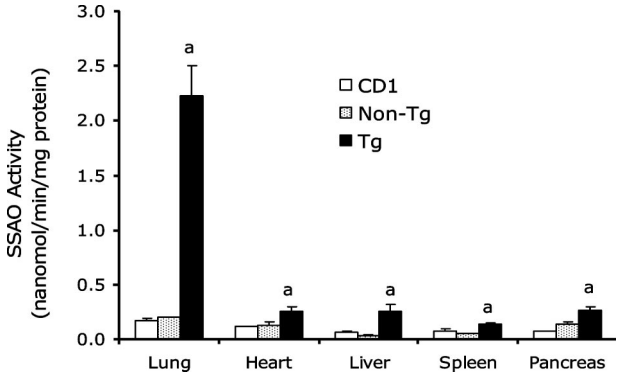

Figure 1. SSAO activity in different tissues from mTIEVAP-1 transgenic mice (Tg), corresponding nontransgenic (non-Tg), and CD-1 Swiss White mice. Data represent mean ± SEM of five animals. $^{a}P < 0.01$, comparison between Tg, non-Tg, and CD1 Swiss white mice.

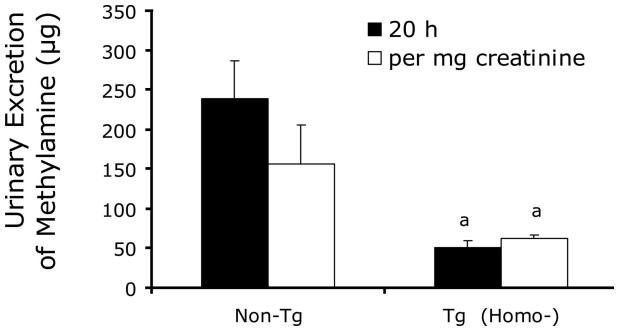

Figure 2. Urinary excretion of methylamine in the transgenic and nontransgenic mice. Both the total amount of methylamine excretion in 20 hours and methylamine levels based on urinary creatinine levels are assessed. Creatinine was determined using the picric acid method. Data represent mean ± SEM of five animals. $^{a}P < 0.01$, comparison between transgenic (Tg) and the corresponding nontransgenic (non-Tg) control mice.

is according to a standard protocol from Panomics. In these experiments transgenic or nontransgenic mice were either pretreated with SSAO inhibitor or saline 2 hours before administration of LPS. BAL fluids were collected 4 hours after LPS treatment. The array membranes were incubated with the BAL fluid (protein concentration, 0.6 mg/ml) and visualized by exposing with Hyperfilm ECL for 1 minute (Amersham Inc., Baie d'Urfe, QC, Canada).

Methylamine Inhalation

Methylamine (200 μl), which is highly volatile, was placed in a small flat dish in a ventilated chamber (12 × 12 × 15 cm). Mice were maintained in the chamber for 1 hour daily for 2 subsequent days and killed on day 3 and BAL fluids were collected for cell counts as described above.

Statistics

The results were assessed using one-way analysis of variance followed by multiple comparisons (Newman-Keuls). The null hypothesis used for all analyses was that the factor has no influence on the measured variable and significance was accepted at >95% confidence level.

Results

SSAO Activity in Different Tissues of Transgenic and Nontransgenic Control Mice

As can be seen in Figure 1, SSAO activities in selected tissues in the mTIEVAP-1 transgenic mice are substantially higher in comparison to the nontransgenic and the CD-1 Swiss White mice. It is interesting that overexpression of human SSAO in these transgenic mice is particularly pronounced (>10-fold) in lung tissues. The TIE-1 promoter was used to target expression of SSAO to endothelial cells of these animals. The lung possesses a high degree of vasculatures that may explain why overexpression of SSAO is more pronounced here than in other tissues.

Methylamine Excretion

Methylamine is an endogenous substrate for SSAO. It is therefore interesting to know whether its excretion is altered in the transgenic mice. As shown in Figure 2, a significant reduction of total urinary excretion of methylamine in 24 hours was observed in the homozygous transgenic mice. The decrease in methylamine excretion was also significant based on creatinine levels. We also observed that the reduction of methylamine excretion was less pronounced in the heterozygous than that in the homozygous transgenic mice.

Deposition of Radioactivity in Tissues after Administration of [^{14}C]-Methylamine

Deamination via SSAO is the primary route of catabolism for methylamine. The product formaldehyde cross-links with tissue constituents and forms long-lasting radioactive protein deposits.[28] As can be seen in Figure 3, after administration of [^{14}C]-methylamine, a significant amount of residual radioactivity was detected in different mouse tissues 72 hours after treatment and complete washout of methylamine. Transgenic mice, which possess higher SSAO activity, produce more formaldehyde and thus

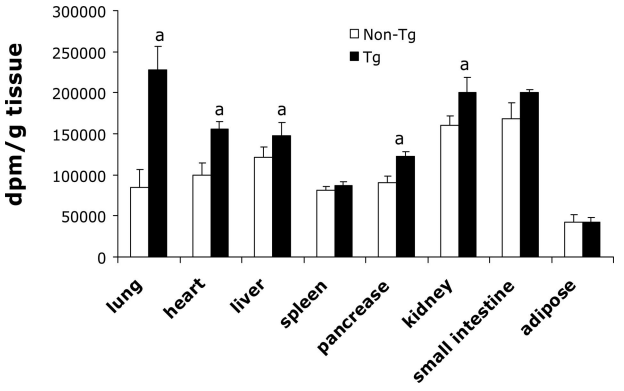

Figure 3. Residual radioactivity in different tissues of transgenic (Tg) overexpressing human SSAO and nontransgenic (non-Tg) mice 72 hours after intravenous injection of [^{14}C]-methylamine. Data represent mean ± SEM of five animals. $^{a}P < 0.01$, comparison of the Tg to non-Tg.

form more radioactive residues in different tissues than that in the nontransgenic mice and another wild-type CD1 Swiss white mice (not shown). Blocking SSAO activity by selective inhibitor significantly reduces the deposition of radioactivity in tissues of the transgenic mice.

Evidence of Increased SSAO-Mediated Protein Formylation in Transgenic Mice

To prove that the formation of protein adducts is a result of SSAO-mediated deamination of methylamine, the amino acid residues of the formaldehyde-protein complexes were assessed. Lung tissue slices from both transgenic and nontransgenic mice pretreated with or without SSAO inhibitor MDL-72974A were incubated with [^{14}C]-methylamine. Labeled protein adducts were reduced by sodium borohydride (to convert the Schiff's base to covalent bond between formaldehyde and proteins) and hydrolyzed in HCl. The amino acid residues were derivatized with FMOC and detected by HPLC/fluorometry. As can be seen Figure 4A, radioactivity present in HPLC fraction 29 was identified to be *N*-methyl-lysine. Radioactivity present in HPLC fractions 14 to 18 are corresponding to *N*-methyl-arginine, *N*,*N*-dimethyl-lysine, and *N*,*N*,*N*-trimethyl-lysine, respectively. SSAO inhibitor MDL-72974A effectively blocks the generation of methylated lysine and arginine. Radioactivity was also detected in fraction number 3. Although MDL-72974A also inhibits the formation of these radioactive products, the identity of these residues is unknown. Figure 4B shows the combined results obtained from four independent animals of transgenic, nontransgenic, and transgenic pretreated with SSAO inhibitor. Lysine residues of proteins are clearly the primary targets for interaction for the SSAO-mediated formaldehyde. Overexpression of lung SSAO clearly enhances the production of formaldehyde-protein adducts.

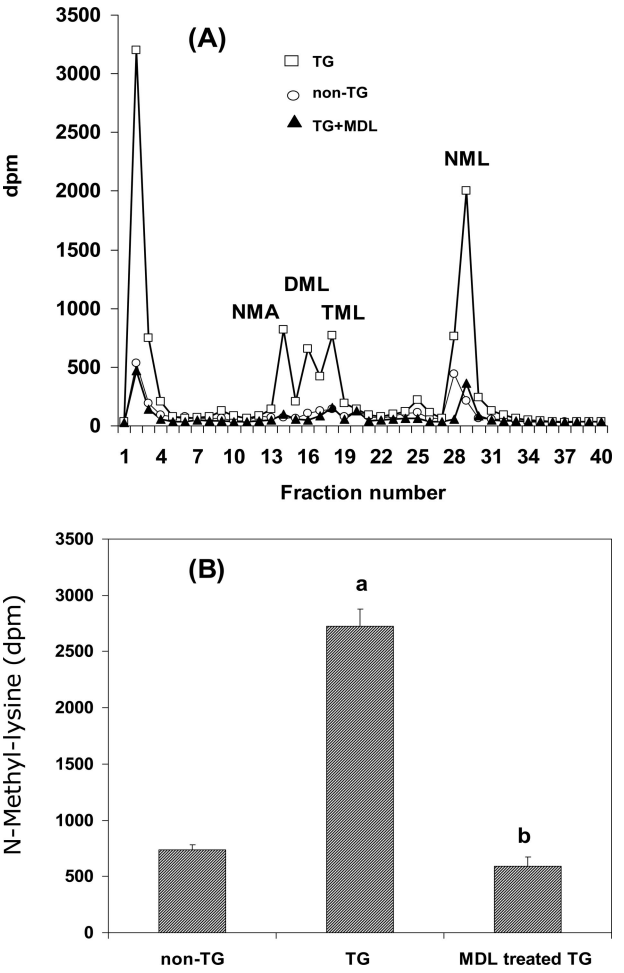

Figure 4. Identification of formaldehyde-protein interaction in lung tissues in transgenic and nontransgenic mice as a result of deamination of methylamine. **A:** Lung tissue slices were incubated with [^{14}C]-methylamine. The formaldehyde-protein adducts were treated with $NaCNBH_3$ before HCl hydrolysis. Amino acids in the hydrolysates were derivatized with FMOC-Cl, separated by HPLC, and assessed by UV detection ($\lambda = 265$ nm). The FMOC derivatives of *N*-methyl-lysine (NML), *N*,*N*-dimethyl-lysine (DML), *N*,*N*,*N*-trimethyl-lysine (TML), and *N*-methyl-arginine (NMA) as indicated are separated from other amino acids. **B:** Data represent mean ± SEM of four independent experiments. $^{a}P < 0.01$, comparison of the untreated Tg and non-Tg and $^{b}P < 0.01$, between treated and untreated Tg.

Increase in BAL Cell Count in Transgenic Mice

The effect of LPS on pulmonary inflammation in both transgenic and nontransgenic mice is shown in Figure 5A. The total BAL cell counts indicate that LPS-instilled mice exhibit an approximately sixfold increase in BAL cells when compared to the sham-exposed nontransgenic control animals. Interestingly, the baseline value of BAL cells in the transgenic mice is significantly elevated in comparison to the nontransgenics. LPS also significantly induces more severe inflammation in the transgenic than the nontransgenic mice. It suggests that overexpression of lung SSAO enhances pulmonary inflammation. Figure 5B shows the typical neutrophils and macrophages in the BAL fluid after Diff-Quick stain. In the LPS-induced lung inflammation the total BAL cells comprised primarily of neutrophils. In the untreated transgenic and nontransgenic mice the cells are primarily macrophages.

Effect of SSAO Inhibitor on LPS-Induced Pulmonary Inflammation

As can be seen in Figure 6A, SSAO inhibitor does not affect the BAL cell counts in untreated nontransgenic animals. However, the inhibitor significantly reduces the LPS-induced increase in BAL cell counts 24 hours after treatment with LPS. These results are consistent with a subsequent analysis of TNF-α levels in BAL fluid (Figure 6B). LPS induces a dramatic increase in BAL TNF-α levels, whereas the SSAO inhibitor MDL-72974A significantly reduces the LPS-induced increase in TNF-α. TNF-α levels are negligibly low in nontransgenic mice with or without pretreatment of SSAO inhibitor. These results support the hypothesis that SSAO is involved in LPS-induced pulmonary inflammation.

The effects of overexpression of SSAO, SSAO inhibitor, and LPS on the expression of several inflammatory cyto-

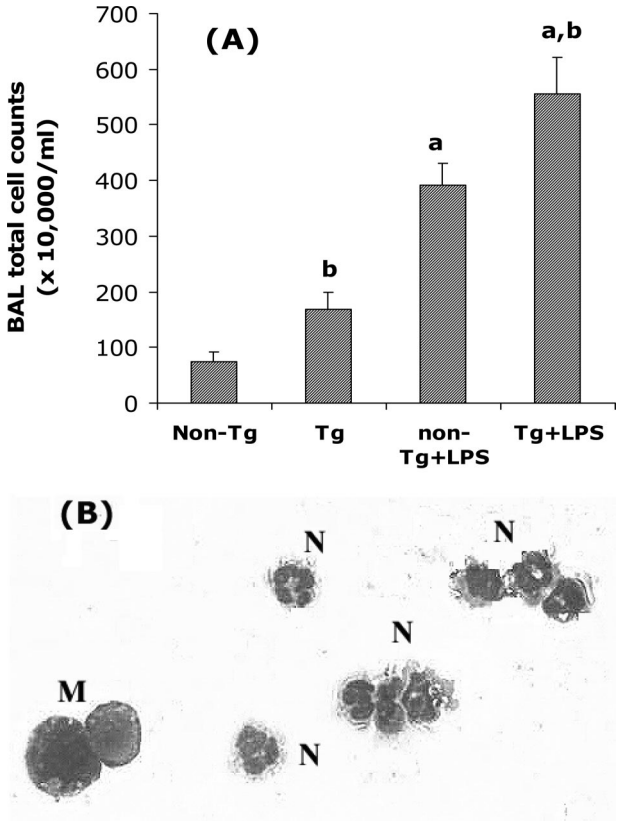

Figure 5. LPS-induced lung inflammation in transgenic and nontransgenic mice. **A:** Ten to twelve transgenic or nontransgenic mice with or without LPS treatment were assessed. Total and neutrophil cells in the BAL fluid were counted. Data represent mean ± SEM of 10 to 11 animals in each experimental group. $^{a}P < 0.01$, comparison of the LPS-treated to corresponding untreated mice and $^{b}P < 0.01$, between Tg and corresponding non-Tg. **B:** Typical view of neutrophil (N) and macrophage (M) in the BAL fluid from a transgenic mouse treated with LPS.

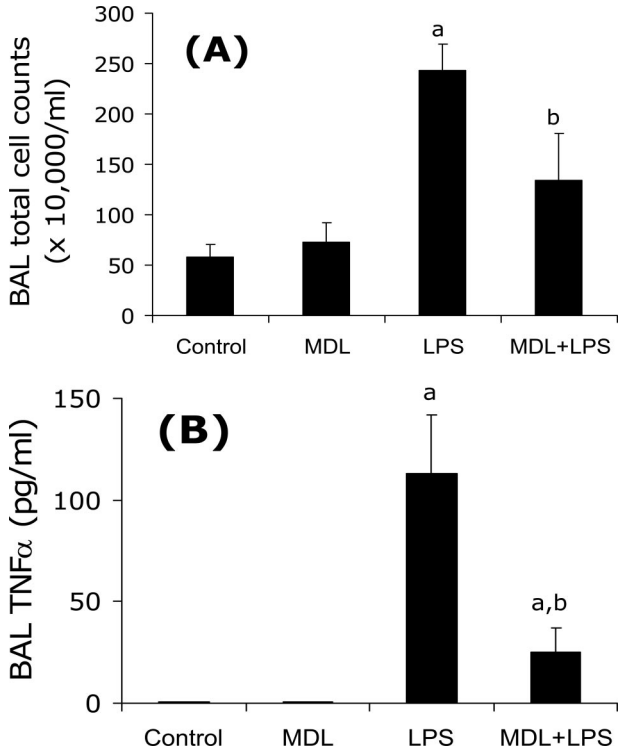

Figure 6. Effect of SSAO inhibitor on LPS-induced lung inflammation in CD-1 mice. **A:** MDL-72974A (5 mg/kg, i.p.) was administered 2 hours before instillation of LPS. BAL fluid was subsequently collected after 24 hours. **B:** TNF-α levels in the supernatant of the BAL fluid was assessed by ELISA. Data represent mean ± SEM of six animals in each experimental group. $^{a}P < 0.01$, comparison of the LPS-treated to corresponding untreated control animals and $^{b}P < 0.01$, between MDL-treated and untreated animals.

kines were assessed using a cytokine antibody array procedure. As can be seen in Figure 7, LPS induces a dramatic increase in macrophage inflammatory protein (MIP)-1α in both transgenic and nontransgenic mice 4 hours after treatment. To a lesser extent an induction of granulocyte colony-stimulating factor (G-CSF) and TNF-α was also detected in the transgenic mice. SSAO inhibitor can significantly reduce the levels of such LPS-induced elevation of these proinflammatory cytokines, such as MIP-1α, G-CSF, TNF-α, IL-6, and so forth. A difference in baseline cytokine levels between the transgenic and nontransgenic mice was not detected using this method.

Effect of Inhalation of Methylamine on Pulmonary Inflammation

To further study the role of SSAO in pulmonary inflammation the transgenic mice were exposed to a methylamine atmosphere. We found that exposure of the lungs to methylamine caused a significant inflammatory response. The total BAL cell counts were significantly increased in the transgenic mice on day 3 after two exposures of methylamine on days 1 and 2 (Figure 8). Such an induction of inflammation was not observed in the nontransgenic mice. Pretreatment with MDL-72974A SSAO inhibitor significantly reduced the methylamine-induced BAL cell numbers. The result clearly shows that inhalation

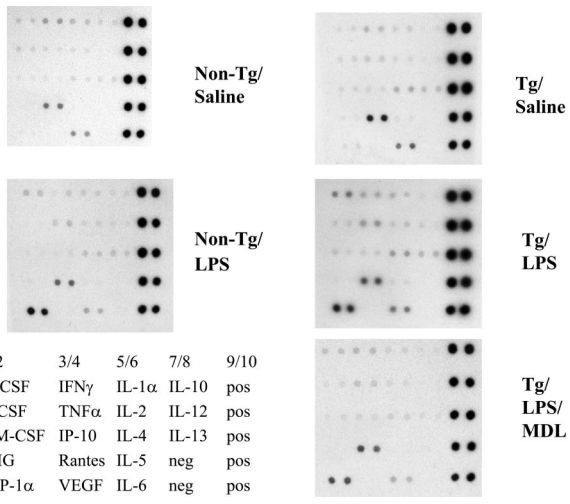

Figure 7. Antibody array analyses of cytokines in BAL fluids obtained from transgenic (Tg) and nontransgenic (non-Tg) treated or untreated with SSAO inhibitor MDL-72974A. Analyses include 16 cytokines: G-CSF, IFN-γ, IL-1α, IL-10, M-CSF, TNF-α, IL-2, IL-12, GM-CSF, IP-10, IL-4, IL-13, MIG, RANTES, IL-5, MIP-1α, VEGF, IL-6. MDL-72974A (5 mg/kg, i.p.) was administered 2 hours before instillation of LPS. BAL fluid was collected after 4 hours thereafter.

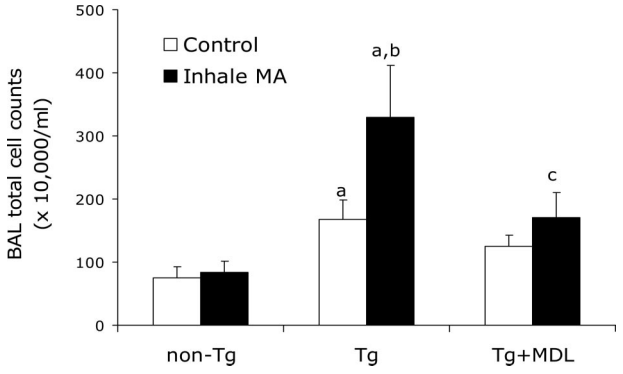

Figure 8. Effect of inhalation of methylamine on pulmonary inflammation. Mice were exposed to methylamine as described in the Materials and Methods section. Data represent mean ± SEM of five animals. [a]$P < 0.01$, comparison of the untreated Tg to untreated non-Tg and [b]$P < 0.01$, between inhalation of methylamine and noninhaled control; [c]$P < 0.01$, between MDL-72974A-treated and untreated animals.

of methylamine and subsequent SSAO-catalyzed deamination can induce acute lung inflammation.

Effect of LPS on Lung SSAO Activity and Formation of Radioactive Residues after Administration of [^{14}C]-Methylamine

As can be seen in Figure 9A, LPS did not significantly alter the lung SSAO activity 48 hours after treatment. SSAO did however contribute to the substantial increase in radioactive residue formation in lung tissue 72 hours after venous injection of [^{14}C]-methylamine (Figure 9B).

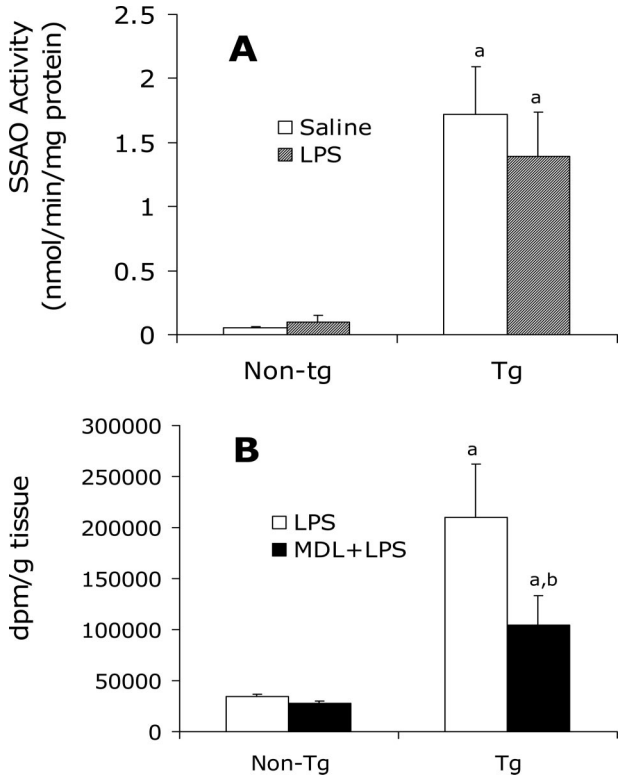

Figure 9. Effect of LPS on lung SSAO activity and residual radioactivity in different tissues after intravenous injection of [^{14}C]-methylamine. **A:** Lung SSAO activity in transgenic and nontransgenic mice was assessed 48 hours after LPS treatment. **B:** The effect of LPS and a SSAO inhibitor on deposition of radioactive adducts in the transgenic mice was determined 72 hours after treatment. Data represent mean ± SEM of six animals in each experimental group. [a]$P < 0.01$, comparison between the transgenic (Tg) and nontransgenic (non-Tg) mice; [b]$P < 0.01$, comparison between MDL-treated and the corresponding controls.

Discussion

SSAO shares some common substrates with monoamine oxidase, a mitochondrial flavine enzyme, but is not inhibited by typical monoamine oxidase inhibitors.[29] Although SSAO has been recognized for a long time little knowledge about its physiological function or even its endogenous substrates has existed. It has now become clear that methylamine and aminoacetone are endogenous substrates for SSAO and the enzyme may be responsible for the development of components in blood vessels,[19] regulation of leukocyte trafficking,[12] and signaling glucose transport in adipocytes.[30] The results presented here further demonstrate that SSAO-mediated deamination may be a critical facilitator of pulmonary inflammation. The LPS-induced increase in BAL cell counts and TNF-α levels estimated by ELISA was found reduced by selective SSAO inhibitor 24 hours after LSP treatment. Because inflammatory cytokines, such as TNF-α and interleukin (IL)-6, are known to increase in BAL as early as 4 hours after endotoxin toxin treatment,[31] we used an antibody array assay to assess 16 different cytokines 4 hours after the treatment of endotoxin. Data are not only consistent with the effect of SSAO inhibitor on TNF-α, but also indicate that LPS-induced increase in MIP-1α and G-CSF levels was much more pronounced and blocking SSAO activity can also reduce the expression of these inflammatory cytokines in the early phase of acute pulmonary inflammation. G-CSF has been considered to be anti-inflammatory and TNF-α and MIP-1α are proinflammatory.[32,33] IL-6 exhibits both proinflammatory and anti-inflammatory activities.[31] Although the details of the inflammatory cascade in the lung remain to be delineated, the involvement of SSAO-mediated deamination in the process is clearly evident. In fact it seems to be an up-stream event of the inflammation process in the present acute inflammatory model.

It is intriguing that deamination of methylamine and aminoacetone by SSAO produces formaldehyde and methylglyoxal, respectively, and hydrogen peroxide, which are all extremely reactive and toxic. It has been shown that increased SSAO-mediated deamination may cause damage to the vasculatures.[21] As SSAO is localized on the outer surface of vascular smooth muscle and endothelial cells, aldehydes produced via deamination are not readily detoxified as in the cells.[1] Furthermore, an increase in serum SSAO activity has been repeatedly found in various pathological conditions associated with vascular disorders, such as in diabetes, obesity, and heart patients.[1] This could be a result of shedding of the enzyme from the inner surface of the blood vessels.[34] This may cause more vascular damage and release of active SSAO related to a chronic toxic cycle and inflam-

mation. The hypothesis is also supported by several animal investigations.[1,28] Formaldehyde and methylglyoxal are capable of cross-linking with proteins following a pseudo-first order kinetic. It is therefore possible that the increase in SSAO-mediated deamination may enhance protein cross-linkage on the surface of the blood vessels and may contribute to the chronic formation of plaques. The protein cross-linkage and aggregation process can be at a subtoxic level, silent and accumulative, and is consistent with the chronic nature of the vascular disorders.

Lung tissues exhibit substantially higher SSAO activities in adult humans than in the rodents, ie, twofold to fourfold.[15,16] The transgenic mice overexpressing SSAO used in the present study exhibit approximately twofold higher SSAO activity in comparison to that in the adult human lungs. They are therefore very useful for the investigation on the role of SSAO in lung inflammation. That inhalation of methylamine induces pulmonary inflammation only in the transgenic mice but not in the nontransgenic mice, suggests that a threshold of SSAO activity is required to contribute to the inflammatory process. Short chain aliphatic amines, such as methylamine, are volatile substrates for SSAO. It is reasonable to propose that inhaled methylamine would be readily deaminated in the lung and become harmful. Indeed, it was reported that an accident spill of methylamine caused severe damage in the lung and respiratory tract, hypotension, and death.[35] Methylamine caused prolonged and robust relaxation of blood vessels and signs of lung inflammation and extensive protein aggregation.[35] This suggests that SSAO-catalyzed production of formaldehyde is involved in the toxic effect of the methylamine. Formaldehyde is well known to be a potent inflammatory agent[36] and widely used in inflammatory pain research.[37,38] Indeed, it is known that formaldehyde is capable of causing upper airway hyperreactivity.[38]

VAP-1 was initially discovered for its role in the recruitment of lymphocytes to the endothelial surface and its expression was found to be up regulated in response to inflammation in several experimental models.[2,13,39] It was subsequently identified to be the same protein as SSAO.[11] Under the present experimental paradigm of LPS/lung inflammation model, no significant induction of lung SSAO activity was detected. However, a significant increase in formation of formaldehyde-protein adducts was detected when [^{14}C]-methylamine was administered. This result suggests that LPS may cause cellular damage and subsequently conformational and/or compartmental changes of the enzyme in favor of deamination of methylamine and thus induces more interactions between formaldehyde and proteins. It has also been reported that VAP-1 (SSAO) resides in intracellular vesicles and can be translocated to the cell surface in animal skin and joint inflammation models.[40] SSAO may also be activated by endogenous SSAO activators, which are known to be present in the lungs.[41]

The present study has been focused on deamination of methylamine. It is anticipated that deamination of aminoacetone, which led to synthesis of methylglyoxal, will also act very much like that of methylamine. Both amines are synthesized endogenously. Other endogenous substrates for SSAO remain to be uncovered. Methylamine is derived from creatine,[42] adrenaline,[43] lecithin,[44] and so forth. Methylamine is also a major component in cigarette smoke.[45] Despite our findings that acute inhalation of high concentrations of methylamine can induce acute pulmonary inflammation, the effect of relatively low concentrations and/or chronic inhalation of methylamine (eg, through tobacco smoke) are unknown. It would be very interesting to know whether such chronic exposure of low dose of methylamine may be related to allergic or nonallergic respiratory disorders. Indeed, our data indicate that the transgenic mice overexpressing human SSAO not only exhibited increased activity in catalyzing the deamination of methylamine, but also substantially enhanced the formation of formaldehyde-protein adducts, which was detected as long-lasting residual deposits. It is possible that these apparently new proteins may trigger immuno-responses related to autoimmunity and inflammation, although our data showed that the inflammation induced by methylamine was less potent than that induced by LPS. The role of SSAO-mediated deamination associated to the inflammatory cascades of the vasculatures seems to warrant further investigation.

In conclusion, evidence indicates that increased SSAO-mediated deamination in transgenic mice overexpressing human SSAO can exacerbate LPS-induced pulmonary inflammation. Blocking SSAO activity can ameliorate such inflammation. Reactive aldehydes, which are generated via deamination of either endogenous or inhaled amine substrates in the lung, may be cytotoxic as well as induce protein cross-linkage leading to autoimmune responses. SSAO, also known to be a cell surface adhesion molecule (VAP-1), is at least partly responsible for recruiting inflammatory cells. This intriguing protein with dual functions may be an alternative target for anti-inflammatory management.

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