Anti-Inflammatory Effects of Inhibiting the Amine Oxidase Activity of Semicarbazide-Sensitive Amine Oxidase

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ABSTRACT

Human semicarbazide-sensitive amine oxidase (SSAO) or vascular adhesion protein-1 (VAP-1) is a copper-containing amine oxidase (AOO3, EC 1.4.3.6) that has both enzymatic and adhesive function. SSAO catalyzes the oxidative deamination of primary amines, resulting in the formation of the corresponding aldehyde and release of hydrogen peroxide and ammonia. Membrane-bound SSAO is an inflammation-inducible endothelial cell adhesion molecule that mediates the interaction between leukocytes and activated endothelial cells in inflamed vessels. Both the direct adhesive and enzymatic functions seem to be involved in the adhesion cascade. LJP 1207 [(N’-(2-phenyl-allyl)-hydrazine hydrochloride] is a potent (human SSAO IC50 = 17 nM), selective, and orally available SSAO inhibitor that blocks both the enzymatic and adhesion functions of SSAO/VAP-1. In a mouse model of ulcerative colitis, LJP 1207 significantly reduces mortality, loss of body weight, and colonic cytokine levels. Quantitative histopathological assessment of colitis activity in this model showed a highly significant suppression of inflammation, injury, and ulceration scores in the animals treated with the SSAO/VAP-1 inhibitor. LJP 1207 also reduced serum levels of tumor necrosis factor-α and interleukin 6 in lipopolysaccharide (LPS)-challenged mice and prolonged survival post-LPS-induced endotoxemia. Therapeutic and prophylactic administration of LJP 1207 in the rat carrageenan footpad model also markedly inhibited swelling and inflammation. Overall, the data suggest that small molecule SSAO/VAP-1 inhibitors may provide clinical benefit in the treatment of acute and chronic inflammatory diseases.

SSAO is a copper-containing amine oxidase that converts primary amines into the corresponding aldehydes while releasing ammonia and hydrogen peroxide (Buffoni and Iginestri, 2000): RCH2NH2 + H2O + O2 → RCHO + NH3 + H2O2. The products of this reaction are notable for their pharmacologic activity. For example, formaldehyde and oxygen free radicals are produced when SSAO processes the physiological substrate methylamine. In mammals, two forms of SSAO have been identified: a tissue-bound form and a soluble plasma form (Lyles, 1996). Several lines of evidence suggest that soluble SSAO originates from the tissue-bound form through proteolytic cleavage (Stolen et al., 2004a). Membrane-bound SSAO is located in the plasma membrane and possesses a single transmembrane portion, a short intracytoplasmic tail, and a large extracellular domain that contains the active center (Li et al., 1998). It is a highly glycosylated dimer with a molecular mass of 180 kDa and contains two subunits and two cupric ions per mole. The active site, containing one Cu2+ and one carbonyl cofactor identified as TPQ connected by a water molecule, is located inside each subunit and communicates with the solvent through a hydrophilic channel (Holt et al., 1998). The TPQ oxygen atoms are hydrogen-bonded to water molecules and/or to the side chain of a conserved amino acid.

In contrast to the better-studied monoamine oxidases (MAO), which metabolize primary, secondary, and tertiary amines, SSAO acts exclusively on primary amines. Even though a number of exogenous and endogenous amines have been identified as in vitro substrates, benzylamine is the most extensively studied substrate for SSAO. Despite considerable advances in the knowledge of SSAO, much remains unknown concerning the structure, mechanism of action, biological substrates, and the general biological function of the enzyme.

ABBREVIATIONS: SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein-1; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; IL, interleukin; TPQ, 2,4,5-trihydroxyphenyl alanine quinone; MAO, monoamine oxidase; ANOVA, analysis of variance; PPO, prostaglandin E2; mPGE1, microsomal PGE synthase-1; ELISA, enzyme-linked immunosorbent assay; LJP 1207, N’-(2-phenyl-allyl)-hydrazine hydrochloride; AM, acetoxymethyl ester.
enzyme. Such lack of knowledge is in part due to the lack of inhibitors having high potency and selectivity.

Cloning of the adhesion molecule vascular adhesion protein-1 (VAP-1) revealed it to be identical to membrane-associated SSAO (Zhang and McIntire, 1996; Smith et al., 1998a). In humans, cell-associated SSAO is expressed mainly in smooth muscle cells of the vasculature, endothelial cells of lymphatic organs, liver sinusoidal tissue, brain microvasculature, and adipocytes. Cell-surface expression of SSAO is tightly regulated and is significantly up-regulated at sites of inflammation (Jaakkola et al., 2000; Salmi and Jalkanen, 2001). SSAO is unique among other endothelial cell-expressed adhesion molecules because of its amine oxidase enzymatic activity. Several studies revealed that SSAO-mediated adhesion and transendothelial migration of leukocytes through endothelial cell layers is mediated by its enzymatic activity and can be blocked by small molecule inhibitors (Salmi et al., 2001). Increases in the levels of both plasma and/or membrane-associated SSAO have been reported for many inflammation-associated diseases, including rheumatoid arthritis, inflammatory bowel disease, type 1 and type 2 diabetes, atherosclerosis, and chronic heart failure (O'Sullivan et al., 2004). The possible involvement of SSAO in the inflammatory processes associated with Alzheimer's disease has also been reported (Yu, 2001).

Recent published work with transgenic mice overexpressing human SSAO also supports the disease associations (Stolen et al., 2004b). Furthermore, studies with genetically modified animals also show that SSAO is the only source of circulating and soluble SSAO activity in the mouse (Stolen et al., 2004b). SSAO-deficient mice are symptom-free in the resting state but display impaired leukocyte adhesion to endothelial cells of inflamed vessels (Stolen et al., 2005), further supporting a role for this molecule in inflammation. The fact that SSAO has both enzymatic and adhesive activities, together with the strong correlation between its up-regulation and many inflammatory conditions, makes it a potential therapeutic target for drug discovery.

In the present study, we utilized a novel, potent, and specific inhibitor of SSAO, LJP 1207, to assess the potential therapeutic benefit of SSAO inhibition in several rodent models of inflammation. Mechanistic studies evaluated the effect of SSAO inhibition on inflammatory parameters at different points of disease development, including cytokines that are known mediators of inflammation. The results demonstrate that SSAO is a major contributor to inflammation, probably by facilitating leukocyte transport into tissues, and support the development of SSAO inhibitors as anti-inflammatory agents.

Materials and Methods

Animal Handling. Animals were purchased from the sources indicated below and kept at a contract vivarium facility (Perry Scientific, San Diego, CA). All protocols were subjected to Institutional Animal Care and Use Committee approval and conform to United States Department of Agriculture requirements. Age- and sex-matched animals were used throughout these studies. Unless otherwise indicated, food and water were supplied ad libitum.

Cloning and Expression of Full-Length Human SSAO/VAP-1. Full-length human SSAO was amplified from a cDNA pool from human lung (Invitrogen, Carlsbad, CA). The 5’ primer for amplification used the sequence 5’-gaa ttc ggg aaa atg aac cag aag-3’, including the restriction site for EcoRI. The 3’ primer had the sequence 5’tct aga cct agt tgt gag agc-3’, including an XbaI restriction site. After 35 cycles of PCR, the 2.3-kb product was purified, digested with EcoRI and XbaI (New England Biolabs, Beverly, MA), and ligated into the mammalian expression vector pcDNA3.1 (Invitrogen) cut with the same enzymes. Top10 Escherichia coli cells (Invitrogen) were chemically transformed with the ligation mixture and spread on Luria broth/carbenicillin plates. Colonies were propagated in Luria broth media, and plasmid DNA was isolated using mini-prep kits (QIAGEN, Valencia, CA). Positive transformants identified by restriction digest were sequenced, and their identity was confirmed as human SSAO, as described by the coding sequence of GenBank accession number NM_003734.2.

Colorimetric Enzyme Activity Assays. MAO-A and MAO-B activities were measured using the coupled colorimetric method essentially as described previously (Holt et al., 1997). Recombinant human MAO-A and human MAO-B enzymes were obtained from BD Biosciences, San Jose, CA). In brief, a predetermined amount of inhibitor diluted in 0.2 M potassium phosphate buffer, pH 7.6, was added to each well of a 96-well microtiter plate. The final concentration of inhibitor was between 50 nM and 1 mM. Controls lacked inhibitor. The following agents were then added to a final reaction volume (200 μl in 0.2 M potassium phosphate buffer, pH 7.6): 0.04 mg/ml MAO-A or 0.07 mg/ml MAO-B enzyme; 15 μl of 10 mM tyramine substrate (for MAO-A) or 15 μl of 100 mM benzylation substrate (for MAO-B); and 50 μl of freshly made chromogenic solution containing 750 nM vanillic acid, 400 nM 4-aminoantipyrine, and 12 U/ml horseradish peroxidase (all from Sigma-Aldrich, St. Louis, MO). Reagents were titrated to give a change of 0.5 OD A490/n, which was within the linear response range of the assay. The plates were incubated for 1 h at 37°C, and the increase in absorbance, reflecting MAO activity, was measured at 490 nm using a microplate spectrophotometer (Power Wave 40; Bio-Tek Instruments, Winooski, VT). Clorgyline and pargyline (inhibitors of MAO-A and MAO-B, respectively) at 0.5 and 10 μM, respectively, were added to some wells as positive controls for MAO inhibition. Inhibition was presented as percent inhibition compared with control after correcting for background absorbance, and IC50 values were calculated from a dose-response curve using Prism software (GraphPad Software Inc., San Diego, CA).

Radiochemical Assay of SSAO Activity. SSAO activity was also measured as described previously (Lizzano et al., 1998). In brief, rat lung or human umbilical cord homogenates were prepared by chopping the freshly excised tissue into small pieces and washing them thoroughly in PBS. The tissue was then homogenized 1:20 (w/v) in 10 mM potassium phosphate buffer, pH 7.8, and centrifuged at 1000g for 10 min at 4°C, and the supernatant was kept frozen until ready to use. SSAO activity in 100 μl of lung homogenate was determined radiochemically using 20 μM [14C]benzylamine as substrate and a determined amount of inhibitor. The reaction was carried out at 37°C in a final volume of 400 μl of 100 mM potassium phosphate buffer, pH 7.2, and stopped with 100 μl of 2 M citric acid. Radioactively labeled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) PPO before liquid scintillation counting. All experiments were performed in the presence of 100 μM pargyline for total inhibition of MAO-B activity in the tissue of homogenates. Inhibition was calculated as percent inhibition compared with control after correcting for background absorbance, and IC50 values were calculated from a dose-response curve using Prism software. Using this method, the inhibitory activity of compounds was also tested in the presence of up to 50% human, rat, and mouse serum.

In Vitro Blocking of Peripheral Blood Mononuclear Cell Adhesion to Rat High Endothelial Cells. Blocking antibodies used in this study included anti-human VAP-1 (clone TK8; Serotec, Raleigh, NC), as well as isotype controls (BD Biosciences). The small molecule blockers semicarbazide, clorgyline (MAO-A inhibitor), and pargyline (MAO-B inhibitor) were all from Sigma-Aldrich. Primary
cultures of high endothelial cells (HEC) were kindly provided by Dr. Ann Ager (Medical Research Council, London, UK) and were established from rat cervical lymph nodes and maintained as described previously (Ager, 1987). HEC were transfected with full-length human SSAO/VAP-1 clone FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to manufacturer’s directions. Positive expression was determined by FACS analysis using the monoclonal primary antibody clone TKS to identify the SSAO protein.

HEC were plated at 3000 cells/well in 96-well plates (Nalgene Nunc, Naperville, IL) in RPMI 1640 medium plus 10% fetal calf serum and incubated overnight at 37°C in a humidified atmosphere of 5% CO2. Nonadherent cells were removed by washing twice the manufacturer’s instructions. The cells were then washed three times in RPMI 1640 + 10% FBS, and T cell enrichment was carried out by negative selection using a mouse CD3+ T cell isolation column (R&D Systems, Minneapolis, MN). LP T cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% NCTC-109, 20 mM HEPES, 2 mM glutamine, 50 μg/ml penicillin/streptomycin, 50 μg/ml gentamicin, 50 μg/ml 2-mercaptoethanol, and 20 U/ml recombinant mouse IL-2 (BD Biosciences). T cells were stimulated by plate-coated anti-mouse CD3 (10 μg/ml clone 2C11; BD Biosciences) and soluble anti-mouse CD28 (1 μg/ml clone 37.51; BD Biosciences). Cells were plated at 2 × 106 cell/ml, and supernatants were harvested after 48 h and frozen at −20°C. IL-4 and IL-5 (Bender Med-Systems, San Bruno, CA) and IL-13 (R&D Systems) concentrations were determined by commercially available specific ELISA assays as per the manufacturer’s recommendations.

The histological analysis was based on colon sections removed at the indicated time points and fixed in 1% formalin (VWR, West Chester, PA). Tissue processing and analysis were performed blindly at a contract laboratory (Pathology Associates, Frederick, MD). In brief, after paraffin embedding, 5-μm sections were cut and stained with hematoxylin and eosin. Three cross-sections were taken from each animal at 1 (section 1), 3 (section 2), and 6 cm (section 3) from the anus. The degrees of ulceration, inflammation (in mucosa, submucosa, serosa, and outer muscular layers), and epithelial injury (including mucosal and submucosal abscesses, submucosal fibrosis, glandular distortion, and mucosal and submucosal edema/hemorrhage) were graded semiquantitatively as 0 for absent, 1 for minimal, 2 for mild, 3 for moderate, and 4 for marked; a total maximum of 4 (ulceration), 16 (inflammation), and 24 (injury).

Oxazolone-Induced Colitis. Oxazolone colitis was induced as described previously (Heller et al., 2002). In brief, 6-week-old SJL/J male mice (18–22 g; Charles River Laboratories, Inc., Wilmington, MA) had a 2 × 2-cm field of abdominal skin shaved and were presensitized by epicutaneous application of 150 μl of 3% oxazolone (4-ethoxymethylene-2-phenyl-2oxazolin-5-one; Sigma-Aldrich) in 100% ethanol on day 0. Five days after skin sensitization, mice were graded semiquantitatively as 0 for absent, 1 for minimal, 2 for mild, 3 for moderate, and 4 for marked; a total maximum of 4 (ulceration), 16 (inflammation), and 24 (injury).

Carrageenan-Induced Paw Edema. Six-week-old female Sprague-Dawley rats (180–200 g; Harlan, Indianapolis, IN) were administered a single compound dose either orally (30 mg/kg LJP 1207 or 3 mg/kg indomethacin) or i.p. (3 mg/kg dexamethasone) at the indicated time points. Rats were fasted 18 h before oral compound administration. The control group was administered an equal volume of vehicle (PBS). Paw edema was induced in anesthetized rats by injecting 50 μl of a 0.5% solution of carrageenan (Type IV Lambda; Sigma-Aldrich) in saline with a 27-gauge needle s.c. in the right hind foot pad. The size of the tested foot of each animal was measured volumetrically using a plethysmometer (Kent Scientific, Torrington, CT) before induction of edema and at different time points after carrageenan injection. At selected times after challenge, rats were asphyxiated with CO2, and their paws were removed. The paws were lacerated with a scalpel, suspended off the bottom of a polypropylene 1.5-ml tube with a micropipette tip, and centrifuged at 10,000 rpm for 10 min, 4°C to express the inflammatory fluid. The volume collected from each paw was determined, and the fluid was analyzed by ELISA for PGE2, IL-1β, and TNF-α production using commercial kits (R&D Systems) according to manufacturer’s instructions.

Relative Quantitative PCR for Cyclooxygenase-2 and Microsomal Prostaglandin E Synthase-1. Right hind paws were excised, and 1 ml of TRIZOL (Invitrogen) was injected into the paw pad. The paws were then placed in a microcentrifuge tube and centrifuged at 10,000g for 2 min. The resulting exudates were collected and made up to 1.5 ml with TRIZOL. Samples were placed on dry ice and stored at −70°C until ready to use. Total RNA was prepared as recommended by the manufacturer. The samples were treated with amplification grade DNase I (Invitrogen). Approximately 1 μg of the DNase-treated total RNA was used for cDNA production using Superscript III with random hexamers (Invitrogen) according to the manufacturer’s protocol. One microliter of the resulting cDNA was used as template for the relative PCR reactions.

COX-2 and microsomal prostaglandin E synthase-1 relative PCR was performed using the Ambion relative competitive PCR system (Ambion, Austin, TX). COX-2-specific primers were as follows: forward primer, 5′-cct gat etg ccc tca cag cgt g-3′, and reverse primer, 5′-gcg caa aga tct gcc ccc c-3′. mPGES-1-specific primers were as follows: forward primer, 5′-ggg aac tgt gcg aga acu c-3′, and reverse
Results

SSAO Inhibitor. LJP 1207 is a small molecule compound containing a hydrazino functional group with a formula weight of 184.67. This compound is a potent and selective inhibitor of SSAO enzymatic activity: rat SSAO IC₅₀ = 7.5 nM; human SSAO IC₅₀ = 17 nM. These potencies were determined in static conditions were evaluated in our study using LJP 1207 and other small molecule inhibitors. The adhesion assay was based on the stable cytoplasmic incorporation of a fluorescent dye, calcine-AM. PBMC are labeled with calcine-AM and collected and frozen until time of cytokine measurements. IL-1β and TNF-α, and IL-6 concentrations were determined by ELISA using commercial kits (R&D Systems) according to manufacturer’s instructions.

Statistics. Unpaired Student’s t test, one-way ANOVA, Dunnett’s test following analysis of variance, and Kaplan Meier survival curves were applied as indicated using Prism software.

Inhibition of SSAO/VAP-1-Mediated Adhesion. Oxazolone-induced colitis has been proposed as a model for human inflammatory bowel disease, especially ulcerative colitis (Strober et al., 2002). Disease severity as measured by body weight loss was maximal at day 7 (2 days after intrarectal challenge), which was also the day when animals start dying (Fig. 2). Intrapерitoneal administration of LJP 1207 twice daily starting on day 0 to mice with oxazolone-induced colitis had a significant effect in preventing body weight loss and improving survival rates (80% survival in the LJP 1207 group versus 40% survival in PBS-treated group at day 11) (Fig. 2). The impact of SSAO inhibition on colon histology was

Fig. 1. SSAO/VAP-1 blockade diminishes the binding of PBMC to HEC. Mock-transfected (A) and SSAO/VAP-1-transfected (B) HEC were pretreated with the indicated blockers before the adhesion assay, as described under Materials and Methods. Binding of calcine-labeled PBMC to the endothelial monolayer was quantitated by measuring fluorescence. MNT, mock-transfected cells, not treated; VNT, SSAO/VAP-1-transfected cells, not treated; VAP, cells treated with anti-VAP-1 antibody (10 µg/ml); 1207a, cells treated with LJP 1207 (10 nM); 1207b, cells treated with LJP 1207 (100 nM); 1207c, cells treated with LJP 1207 (10 µM); Semic, cells treated with semicarbazide (500 µM); Clog, cells treated with clorgyline (250 µM); Parg, cells treated with parargyline (200 µM). Results are the mean ± S.D. of three experiments with six samples per experiment and are expressed as percentage of baseline binding of PBMC to MNT cells (100%, dotted line).
assessed in mice sacrificed at day 7. This model displays higher disease severity in the distal colon (sections 1 and 2), as opposed to other colitis models where injury and inflammation occur closer to the cecum. As shown in Fig. 3, quantitative histopathological assessment of colitis activity in these animals showed a highly significant suppression of inflammation, injury, and ulceration scores in section 2 (3 cm from anus) in mice treated with LJP 1207. The total maximal cumulative histological score for each section as defined in this study was 44 (4 for ulceration, 16 for inflammation, and 24 for epithelial injury; see Materials and Methods). Mean total cumulative scores for section 2 were 19.4 and 1.2 in the colons of animals administered PBS and LJP 1207, respectively. Histological scores for section 1 (1 cm from anus) showed a relative difference between treatment groups equivalent to the one observed for section 2. Section 3 (6 cm from anus) did not show significant disease progression in any of the treatment groups. As mentioned, in this model, disease is initiated in the lower colon and progresses toward the cecum; by day 7, disease is still relatively early in development.

Oxazolone-induced colitis in SJL/J mice is a T helper cell-2-mediated model, where T cells from lesional tissue produce markedly increased amounts of IL-4, IL-5, and IL-13 (Heller et al., 2002). This response is counterbalanced by tumor growth factor-β (Boirivant et al., 1998). Thus, cytokine levels were monitored at different time points after disease induction in order to investigate whether SSAO inhibition has an impact on this underlying mechanism of disease.

Colons from animals dosed twice daily with PBS or 10 mg/kg LJP 1207 were collected 2 and 6 days after intrarectal injection (days 7 and 11, respectively) and assayed for T cell-derived cytokine profiles. Mononuclear cells were isolated from lamina propria, and T cells were isolated and cultured in the presence of anti-CD3 antibody, anti-CD28, and IL-2. Supernatants were harvested after 48 h and assayed for the presence of IL-4, IL-5, and IL-13 by ELISA. Data for day 7 cytokine levels showed that LJP 1207 had a major impact on the amount of IL-4, IL-5, and IL-13 being produced (Fig. 4). The effect of SSAO inhibition on cytokine levels is lost by day 11, mainly because the animals that survived up to that point (11/11 in LJP 1207 group and 8/11 in PBS group) had low absolute levels of cytokines produced by colonic T cells as they were recovering. Hence, the low absolute levels of cytokines were produced by colonic T cells at this time point.

SSAO Inhibition in Acute Inflammation. The carrageenan-induced paw edema model of acute inflammation allows for evaluation of inflammatory swelling secondary to COX-2-produced prostaglandin (PG; Seibert et al., 1994; Smith et al., 1998b). To assess the therapeutic effect of SSAO inhibition in the inflamed footpad, compounds were orally

![Fig. 2.](image-url)  
LJP 1207 prolongs survival and reduces body weight loss in mice with oxazolone-induced colitis. Animals were presensitized by skin painting with 3% oxazolone on day 0 and were intrarectally challenged 5 days later with 1% oxazolone in 50% EtOH. LJP1207, animals dosed with 10 mg/kg LJP 1207; PBS, animals dosed with PBS (both groups dosed i.p. twice daily starting on day 0); ETOH control group, animals presensitized by skin painting with 3% oxazolone and, 5 days later, intrarectally challenged with 50% ETOH only (n = 8).

![Fig. 3.](image-url)  
Histological assessment of colitis in mice with oxazolone-induced colitis two days after intrarectal administration of 1% oxazolone (day 7). Colons were fixed and stained with hematoxylin and eosin. Treatment with LJP 1207 (20 mg/kg/day) or PBS was initiated on day 0. A, graphs represent cumulative results from section 2 of all colons. B, section of colon from mouse treated with PBS (100×). C, section of colon from mouse treated with LJP 1207 (100×). Data are shown as mean ± S.E.M. (n = 10). Statistical analysis was performed using one-way ANOVA followed by Dunnett’s test; †, p < 0.01.
administered to animals one h after carrageenan injection. LJP 1207 significantly reduced swelling induced by carrageenan injection, even when administered after inflammation onset. The magnitude of reduction in swelling mediated by LJP 1207 is equivalent to indomethacin for the doses tested (Fig. 5).

To further assess the effect of SSAO inhibition on local inflammatory mediators, three groups were exposed to 30 mg/kg LJP 1207, 3 mg/kg indomethacin, or PBS p.o. 1 h before carrageenan injections. The fourth group was administered 3 mg/kg dexamethasone i.p. 1 h before paw inflammation. The levels of PGE\textsubscript{2}, TNF-\textalpha, and IL-1\beta, which are the primary inflammatory mediators in this model, were evaluated in paw exudates 1, 3, and 6 h after carrageenan injection.

Carrageenan injection into the footpad typically induces a 5- to 10-fold increase in PGs. As expected, dexamethasone was more effective at preventing swelling, whereas indomethacin had a bigger impact on PGE\textsubscript{2} levels (Fig. 6). The level of edema in the LJP 1207 group is comparable with the indomethacin group, and LJP 1207 significantly reduces PGE\textsubscript{2} production to levels equivalent to the ones observed in the dexamethasone-treated animals. Both LJP 1207 and dexamethasone treatments had an impact on TNF-\textalpha at the 1-h time point (TNF-\textalpha levels in this model peak between 60 and 90 min), and as expected, indomethacin did not. Whereas edema was still substantially reduced for all treatments at the 6-h time point, PGE\textsubscript{2} and TNF-\textalpha levels were not significantly affected at this time point (data not shown). With the exception of dexamethasone at 6 h, none of the treatments had a significant effect on IL-1\beta levels.

PG production in this model is driven by COX-2 (Smith et al., 1998b), and LJP 1207 IC\textsubscript{50} for COX-2 is 29 \mu M (1000-fold higher than that for SSAO). Thus, it is unlikely that the effect observed with LJP 1207 is due to cross-reactivity with this enzyme. Alternatively, the effect of LJP 1207 on PG production might be exerted by attenuating TNF-\textalpha, which is a known stimulus for phospholipase A2. To further investigate the role of LJP 1207 on paw swelling in general and PGE\textsubscript{2} production in particular, we examined local mRNA levels for COX-2 and mPGES-1, the primary PGE synthase that contributes to the pathogenesis in several animal models of inflammation. Local COX-2 and mPGES-1 levels in the paws were determined by relative PCR using 18S ribosomal
RNA as an internal control. Quantitative densitometry analysis of the different bands provides the relative gene-of-interest:ribosomal RNA ratios between samples. Using this procedure, we isolated RNA from the test paws of all of the animals and performed individual PCR reactions for each one of them. The mean ± S.D. values for relative mRNA ratios are shown in Fig. 7. Consistent with reported data, indomethacin does not impact levels of COX-2 and mPGES-1 transcripts, whereas dexamethasone does. LJP 1207 significantly reduced the levels of COX-2 and mPGES-1 mRNA expression when compared with placebo. Taken together, these results suggest that LJP 1207 is having an impact in the levels of inflammatory mediators that are driving the edema.

**SSAO Inhibition in Systemic Inflammation.** Exposure of endothelial cells to elevated levels of LPS and inflammatory cytokines leads to up-regulation of adhesion molecules and chemokines, which results in an increase in the tethering, rolling, and transmigration of leukocytes across vascular barriers (Sharma et al., 2004; Villa and Ghezzi, 2004). LPS-induced endotoxemia is a well characterized model of systemic inflammation that is suitable to investigate the putative role of SSAO in some of these mechanisms.

LPS-induced endotoxemia, if untreated, produces an uncontrolled TNF response that causes shock and tissue injury. To assess the role of SSAO inhibitors on the levels of circulatory cytokines, 30 mg/kg LJP 1207 was administered 60 min before an i.p. injection of 5 mg/kg LPS. Dexamethasone was administered i.p. at a concentration of 3 mg/kg 1 h before disease induction. LJP 1207 significantly reduced the levels of circulatory TNF-α (Fig. 8). Both dexamethasone and LJP 1207 also had a significant effect on IL-1β and IL-6 production in this model.

Serum TNF and IL-1β reach toxic levels in mice and human volunteers within 1 to 2 h after LPS administration, and delayed treatment with anti-TNF-α or anti-IL-1β fails to prevent late endotoxemia deaths (Tracey et al., 1987; Fink, 1995). To examine the role of SSAO inhibition in this setting, mice were administered 2 mg/kg LPS together with 300 mg/kg D-galactosamine followed by oral dosing of LJP 1207 at the indicated time points. LJP 1207 consistently prolonged the survival of mice post-LPS shock (Fig. 9).

**Discussion**

LJP 1207 is an orally active, potent, and selective inhibitor of the adhesion molecule SSAO. In vitro studies demon-
strated that a compound that inhibits the amine oxidase activity of SSAO also blocks SSAO-mediated cell adhesion. Its specificity for SSAO has been established against panels of enzymes and receptors. In vivo studies have established efficacy in multiple disease models, including inflammatory bowel disease, local inflammation, and systemic inflammation. Histological and cytokine studies suggest that SSAO inhibition by LJP 1207 has an impact on the local and systemic concentration of inflammatory mediators that drive disease in these models.

Our adhesion studies confirmed that blocking the enzymatic catalytic site has an effect on the adhesion function of this molecule. LJP 1207 reduced binding in a dose-dependent manner of PBMC to HEC transfected with full-length SSAO, down to levels observed in the mock-transfected cells. These results confirm that blocking SSAO enzymatic function with LJP 1207 significantly reduces the binding of PBMC to SSAO-expressing HEC in vitro. Notably, the induction of SSAO expression in HEC markedly increased the expression of the endothelial cell-specific adhesion molecules CD31 and P-selectin (CD62P), whereas intercellular adhesion molecule-1 expression was reduced (data not shown). SSAO-induced reduction in intercellular adhesion molecule-1 expression has also been reported by Salmi et al. (2000) in a similar cell system. Whether or not these results are of physiological relevance or are an artifact of the transfected cells remains to be determined. However, if SSAO does influence cell surface expression of other EC adhesion molecules in vivo, it may be very significant, especially because SSAO cell surface expression is itself tightly regulated and restricted to inflamed vessels (Jaakkola et al., 2000).

Oxazolone-induced colitis in SJL/J mice is caused by a polarized T helper cell-2 T cell response (Strober et al., 2002). This response results in a superficial inflammation that affects the distal part of the colon, maintains villous architecture, and is associated with bowel edema and luminal exudates (Boirivant et al., 1998). Therapeutic administration of LJP 1207 to animals sensitized and challenged with oxazolone caused a profound beneficial effect on the development of disease as illustrated by the favorable impact of LJP 1207 on all of the histological parameters examined. Furthermore, LJP 1207 decreased LP T cell-derived secretion of IL-4, IL-5, and IL-13. The cytokine response of oxazolone colitis has been well studied. According to Strober et al. (2002), oxazolone colitis leads to production of IL-4, which in turn leads to the secretion of IL-13 and IL-9. IL-13 is mainly produced by natural killer T cells and is essential for disease progression (Heller et al., 2002). They propose that IL-13-producing natural killer T cells act as effector cells in this and other murine models of inflammation and may be also involved in the pathogenesis of human ulcerative colitis. Because LJP 1207 significantly reduced the levels of IL-13, as well as IL-4 and IL-5, our data are consistent with the hypothesis that SSAO inhibition is having an impact early on in the underlying mechanism of disease induction.

SSAO inhibition by a small molecule inhibitor is efficacious both as a prophylactic and as a therapeutic agent in the treatment of an aggressive and rapid inflammatory response, as illustrated here in the carrageenin-induced paw edema model. Oral administration of LJP 1207 blocked edema and reduced levels of several local inflammatory mediators, including PGE\textsubscript{2} and TNF-\alpha. The COX enzymes (COX-1 and COX-2) catalyze the oxygenation of free arachidonic acid to the unstable intermediary prostaglandin H\textsubscript{2}. The COX-1/COX-2-nonspecific inhibitor indomethacin had a profound effect on the local and systemic inflammatory response to oxazolone.
impact on PGE$_2$ levels, but it did not alter local expression of TNF-α and IL-1β. These results are in agreement with a large body of work that indicates that blockade of PGE$_2$ per se is not sufficient to produce a full range of anti-inflammatory activities. Moreover, COX-1-derived PGs may not mediate inflammation in the carrageenan paw edema model (Portanova et al., 1996) and selective inhibition of COX-2 only partially reduces the total levels of PGE$_2$ (Smith et al., 1998b). The anti-inflammatory property observed with dexamethasone treatment in this model are well described and was expected, considering that glucocorticoids exert their effects by interfering with several key molecular pathways, including the production of proinflammatory cytokines, expression of adhesion molecules, inhibition of inducible nitric-oxide synthase, and inhibition of COX-2, among other effects. In contrast to the effects of indomethacin, SSAO inhibition has an effect on production of both PGE$_2$ and TNF-α, suggesting that its mechanism of action is distinct from the COX inhibitors.

mPGES-1 is one of several enzymes capable of converting prostaglandin H$_2$ into PGE$_2$. MPGES-1 is inducible after exposure to proinflammatory stimuli (Jakobsson et al., 1999) and is probably responsible for the production of PGE$_2$ during inflammatory responses (Trebin et al., 2003). Although the kinetics of COX-2 and mPGES-1 expression are different (Ivanov et al., 2002; Kojima et al., 2002), they both have a major role in acute PGE$_2$-dependent experimental models of inflammation. Indomethacin, which is known not to influence levels of COX-2 mRNA in leukocytes, also does not have an impact on the transcription of mPGES-1. In contrast, both dexamethasone and LJP 1207 reduced the expression of both COX-2 and mPGES-1 in the affected paws of experimental animals.

Both the enzymatic and adhesive functions of SSAO have been implicated in the trafficking and infiltration of leukocytes into inflamed tissues (e.g., Martelius et al., 2004; Yegutkin et al., 2004). Taken together these results further suggest that adhesive and enzymatic activities of SSAO are pivotal in acute inflammation, especially in the early stages of carrageenan-induced paw edema. The selective reduction of protein levels and gene expression of inflammatory mediators by LJP 1207 suggests an alternative, possibly complimentary pathway, to reduce inflammation that is distinct from nonsteroidal anti-inflammatory drugs.

Oral administration of LJP 1207 prolonged survival subsequent to LPS-induced endotoxemia while reducing serum levels of TNF-α, IL-1β, and IL-6 in LPS-challenged mice. LPS is known to directly regulate expression of adhesion molecules on leukocytes or endothelial cells, some of which can be up-regulated within minutes after exposure to LPS-induced cytokines (Spertini et al., 1991; Coughlan et al., 1994). Inhibition of SSAO may reduce the extravasation and subsequent activation of leukocytes, thereby reducing circulating levels of proinflammatory cytokines and increased survival. Inhibiting the ability of SSAO to convert endogenous amines, such as methylamine, to vasoactive metabolites may also contribute to the beneficial effect of LJP 1207 in this model (Conklin et al., 2004). The timing of therapeutic intervention in LPS-induced endotoxemia is critical to the clinical outcome. For example, antibodies to TNF-α are effective only upon prophylactic administration (Wang et al., 2004). We found that mice treated with LJP 1207 at $T_0$ and at $T + 6$ h had improved survival rates, suggesting that, in addition to lowering initial TNF-α levels, SSAO may also have a “late-acting” function that could be relevant to the use of SSAO inhibitors in the treatment of sepsis and septic shock.

The complete mechanism by which SSAO inhibition interferes with inflammation is not well understood. The ability of a small molecule enzyme inhibitor to block cell adhesion suggests that the active site of the enzyme is directly involved in the adhesion interaction. The byproducts of the SSAO enzymatic reaction also have the potential to modulate vasoactive tone and extravasation. Although there are enzymes that have been implicated in cell adhesion, such as the ADP-ribosyl cyclase CD38, these enzymes do not seem to be related to SSAO.

Several lines of evidence suggest that SSAO is an attractive therapeutic target. First, SSAO is up-regulated in inflamed vessels where it mediates adhesion and transmigration of leukocytes. Therefore, drugs that target SSAO should have preferential effects at sites of inflammation. Second, SSAO is an important contributor to leukocyte transport across vascular barriers. The data reported here, as well as results obtained in SSAO knock-out mice where a 71% reduction in transmigration of polymorphonuclear leukocytes was observed in response to acute TNF challenge (Stolen et al., 2005), thus confirm that SSAO is a major contributor to leukocyte transport in inflammation and explain the profound anti-inflammatory effect of the SSAO inhibitors. Finally, SSAO is readily amenable to an orally administered small molecule approach. Because the adhesive function of SSAO is intimately related to the enzymatic function, small orally active drug-like molecules can readily inhibit both activities. In summary, nonclinical efficacy data from a variety of disease models suggest that SSAO-based antiadhesive therapy may have clinical utility in a wide spectrum of diseases where inflammation contributes to disease pathology.

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References


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