Effects of p38 MAPK Inhibition on Early Stages of Diabetic Retinopathy and Sensory Nerve Function

Yunpeng Du,¹,² Jie Tang,¹,² Guanyuan Li,¹,²,³ Liliana Berti-Mattera,¹ Chieb Allen Lee,¹,⁴ Darian Bartkowski,⁵ David Gale,⁵ Joe Monahan,⁶ Michael R. Niesman,⁵ Gordon Alton,⁵ and Timothy S. Kern¹,⁴

PURPOSE. p38 mitogen-activated protein kinase (MAPK) is known to play a regulatory role in inflammatory processes in disease. Inflammation has been linked also to the development of diabetic retinopathy in rodents. This study was conducted to evaluate the effect of a p38 MAPK inhibitor on the development of early stages of diabetic retinopathy in rats.

METHODS. Streptozotocin-diabetic rats were assigned to two groups—treated with the p38 MAPK inhibitor PHA666859 (Pfizer, New York, NY) and untreated—and compared with age-matched nondiabetic control animals.

RESULTS. At 2 months of diabetes, insulin-deficient diabetic control rats exhibited significant increases in retinal superoxide, nitric oxide (NO), cyclooxygenase (COX)-2, and leukostasis within retinal microvessels. All these abnormalities were significantly inhibited by the p38 MAPK inhibitor (25 mg/kgBW/d). At 10 months of diabetes, significant increases in the number of degenerate (acellular) capillaries and pericyte ghosts were measured in control diabetic rats versus those in nondiabetic control animals, and pharmacologic inhibition of p38 MAPK significantly inhibited all these abnormalities (all P < 0.05). This therapy also had beneficial effects outside the eye in diabetes, as evidenced by the inhibition of a diabetes-induced hypersensitivity of peripheral nerves to light touch (tactile allodynia).

CONCLUSIONS. p38 MAPK plays an important role in diabetes-induced inflammation in the retina, and inhibition of p38 MAPK offers a novel therapeutic approach to inhibiting the development of early stages of diabetic retinopathy and other complications of diabetes. (Invest Ophtalmol Vis Sci. 2010; 51:2158–2164) DOI:10.1167/iovs.09-3674

Disclosure: Y. Du, Pfizer (F); J. Tang, Pfizer (F); G. Li, Pfizer (F); L. Berti-Mattera, Pfizer (F); C.A. Lee, Pfizer (F); D. Bartkowski, Pfizer (F); D. Gale, Pfizer (F); J. Monahan, Pfizer (F); M.R. Niesman, Pfizer (F); G. Alton, Pfizer (F); T.S. Kern, Pfizer (F).

Corresponding author: Timothy S. Kern, Case Western Reserve University, 434 Biomedical Research Building, 10900 Euclid Avenue, Cleveland, OH 44106; tsk@case.edu.

Hyperglycemia causes metabolic and physiologic abnormalities in the retina that can lead eventually to degeneration or dysfunction of the retinal blood vessels and neurons. Diabetes-induced molecular and physiologic alterations consistent with inflammation observed in retinal tissue or cells include upregulation of iNOS, COX-2, ICAM-1, caspase 1, VEGF, and NF-κB; increased production of nitric oxide, prostaglandin E2, IL-1β, and cytokines; and increased permeability and leukostasis. 1,2 Inhibition of this inflammatory cascade at any of its multiple steps can inhibit the histopathologic characteristic of the early stages of diabetic retinopathy in animals. 1,3 These findings have suggested a role of inflammation in development of retinopathy. 1

A large body of evidence in preclinical studies indicates a crucial role of p38 MAPK in inflammation, and p38 MAPK inhibitors have been shown to block production of IL-1, TNF, and IL-6 in vitro and in vivo in other diseases. The p38 MAPK pathway is also involved in the induction of other inflammatory molecules, such as COX-2 and iNOS, 4–6 and p38-dependent phosphorylation of histone H3 has been shown to regulate NF-κB, also resulting in increased expression of inflammatory cytokines and chemokines. 7 Suppression of p38 MAPK has been found to inhibit acute lung inflammation, 8,9 ischemia/reperfusion, 10 rheumatoid arthritis, Alzheimer’s disease, and inflammatory bowel disease, 11–13 as well as in ocular ischemia-reperfusion injury. 14

The demonstrated role of p38 MAPK in the development of inflammation in a variety of diseases makes this protein an attractive target for pharmacologic intervention. Thus, we used the potent and bioavailable p38 MAPK inhibitor PHA666859 (Pfizer, New York, NY) to investigate the possibility that p38 MAPK plays an important role in the pathogenesis of early stages of diabetic retinopathy in a rat model. In the same animals, we also investigated the possibility that p38 MAPK plays a role in the hypersensitivity to light touch (tactile allodynia) that causes some diabetic patients pain and discomfort. 15

MATERIALS AND METHODS

Animals

Male Lewis rats (200–225 g) were randomly assigned to become diabetic or remain nondiabetic, with two study durations: 2 months and 10 months after diabetes induction. Diabetes was induced by intraperitoneal injection of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 55 mg/kg of body weight. Diabetic animals (nonfasting blood glucose >275 mg/dL 2 weeks after injection of streptozotocin) were assigned randomly to receive the p38 MAPK inhibitor or to remain untreated as diabetic control subjects. Drug administration was not begun until 2 weeks after streptozotocin, to ensure that all animals were satisfactorily diabetic and that the drug did not influence the severity of diabetes achieved. All animals were fed a...
Table 1. Effect of PHA666859 on Diabetes Severity over the 10 Months of the Study

<table>
<thead>
<tr>
<th>Duration (mo)</th>
<th>Final BW (g)</th>
<th>24-h Urine Volume (mL)</th>
<th>Average GHB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic (N)</td>
<td>10</td>
<td>643 ± 43</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>Diabetic (D)</td>
<td>10</td>
<td>286 ± 26†</td>
<td>5.8 ± 0.8†</td>
</tr>
<tr>
<td>D+PHA666859</td>
<td>10</td>
<td>293 ± 19†</td>
<td>11.0 ± 1.1†</td>
</tr>
</tbody>
</table>

* Average in each animal over the entire duration of the study.  † P < 0.05 compared with N control.  ‡ P < 0.05 compared with D control.

Two-Month Outcome Measures

Superoxide Anion. Fresh retinas from animals killed at 2 months of study were incubated in 0.5 mM lucigenin, and the luminescence was detected as reported previously.17 The superoxide scavenger tiron inhibits the diabetes-induced increase in luminescence by more than 90%,17 demonstrating that most of the luminescence induced in retinas from diabetic animals is due to superoxide production.

Western Blot Analysis. Retinal homogenates were subjected to Western blot analysis, as reported by us previously.18,19 Antibodies against INOS (mAb; 1:500 dilution; Transduction Laboratories, Lexington, KY), nitrotyrosine (polyclonal; 1:1000 dilution; Upstate Biotechnology, Lake Placid, NY), COX-2 (polyclonal; 1:500 dilution; Cayman Chemical, Ann Arbor, MI), ICAM-1 (1:250 dilution; R&D Systems, Minneapolis, MN) were used. Antibodies against PARS activity (1:400 dilution), pp38 MAPK (threonine 180 and tyrosine 182; 1:1000 dilution), heat shock protein 27 (HSP27; 1:1500 dilution), p-HSPP27 (ser182; 1:1000 dilution), BAD (ser112; 1:1000 dilution), MEK (monoclonal; 1:1000 dilution), p- MEK (ser217 and ser221; 1:1000 dilution), mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 (polyclonal; 1:1000 dilution), and p-MAPKAP kinase-2 (polyclonal; threonine 222; 1:1000 dilution) were all obtained from Cell Signaling Technology, Inc. (Danvers, MA).

Leukostasis. Leukostasis was measured at 2 months, by using published methods.19–21 Briefly, anesthetized rats had a 20-gauge perfusion needle inserted into the base of the aortic arch, making sure that the needle did not obstruct the carotid arteries. The right atrium was cut to allow outflow, and PBS (60 mL) was perfused into the aorta at the normal cardiac output rate for a rat (60 mL/min) to clear erythrocytes and nonadherent leukocytes. Fluorescein isothiocyanate-coupled Concanavalin A lectin (20 μg/mL in PBS [pH 7.4]; total concentration 5 mg/kg body weight; Vector Laboratories, Burlingame, CA) then was perfused to stain adherent leukocytes and vascular endothelium, followed by another wash with PBS (60 mL) at the same perfusion rate to remove excess Concanavalin A. The retina was flatmounted on a microscope slide, covered with antifade medium and a coverslip, and imaged via fluorescence microscopy. The total number of adherent leukocytes per retina was counted.

Ten-Month Outcome Measures

Quantitation of Retinal Disease. The retinal vasculature was isolated from formalin-fixed tissue using the trypsin digest technique.19,22,25 After the purified vessel network was dried onto a glass slide, the preparations were stained with hematoxylin and periodic acid-Schiff, dehydrated, and coverslipped. Acellular capillaries were quantitated in four to seven field areas in the mid retina (200× magnification) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length and were reported per square millimeter of retinal area. Pericyte ghosts were estimated from the prevalence of protruding “bumps” in the capillary basement membranes from which pericytes had disappeared. At least 1000 capillary cells (endothelial cells and pericytes) in five field areas in the mid retina (400× magnification) were examined.

Table 2. Potency and Selectivity of PHA666859

<table>
<thead>
<tr>
<th>Kinase</th>
<th>PHA666859 (IC_{50}, μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P38α MAPK</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>P38β MAPK</td>
<td>2.45 ± 0.54</td>
</tr>
<tr>
<td>P38γ MAPK</td>
<td>&gt;200</td>
</tr>
<tr>
<td>JNK1</td>
<td>7.20 ± 0.20</td>
</tr>
<tr>
<td>JNK2</td>
<td>6.02 ± 3.7</td>
</tr>
<tr>
<td>JNK3</td>
<td>12.3 ± 4.4</td>
</tr>
<tr>
<td>P38α MAPK</td>
<td>K_i = 53 ± 5 nM</td>
</tr>
<tr>
<td>JNK2</td>
<td>K_i = 1780 ± 110 nM</td>
</tr>
</tbody>
</table>

PHA666859 IC_{50} >200 μM: ERK2, PRAK, MSK, MK2, MK3, MNK, M KK6, CDK2, IKK1, and IKK2; PHA666859 IC_{50} >10 μM: PKCβ, PKA, NIM1, EGFR1, LCK, Met, FGRF1, Lyn, Ab1, CHK1, GSK3β, IGFRI, IRAK4, CDK2, and Aurora2.
in a masked manner. Ghosts on any already acellular vessel were excluded.

**Neurodegeneration.** Optic nerves were fixed in 2% glutaraldehyde to count the number of axons in the optic nerve (as a way to quantitate all ganglion cells in the retina). The fixation of this heavily myelinated tissue was not adequate, and as a result, we were not able to count axons in the center of the nerve. Thus, this measurement was regarded as unsuccessful (data not shown).

**Tactile Allodynia.** A standardized testing regimen was performed to measure tactile allodynia, as reported previously by us. Von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% mechanical threshold for foot withdrawal. Lifting of the paw was recorded as a positive response, and the next lightest filament was chosen for the next measurement. Absence of a response after 5 seconds prompted the use of the next filament of increasing weight. All measurements were performed by an investigator who was unaware of the treatment group of individual animals.

**Quantitation of PHA666859**

The drug concentration was quantified by rat plasma using high-performance liquid chromatography–tandem mass spectroscopy (LC/MS/MS). Standard stock solutions of PHA666859 were prepared in acetonitrile and diluted to the working standard solutions (in acetonitrile). The internal standard used was buspirone, which was prepared in methanol (50 ng/mL). All standard and QC stocks were stored at −20°C.

Proteins were precipitated with acetonitrile/methanol (4:1, vol:vol) containing 0.1% formic acid. The LC-MS/MS system consisted of pumps (model LC10AD; Shimadzu, Columbia, MD), with an autosampler (CTC-PAL; LEAP Technologies, Carrboro, NC) and a cool stack and triple quadrupole mass spectrometer (Sciex API 4000; Applied Biosystems, Inc. [ABI], Foster City, CA). A gradient elution method was used to perform chromatography on a reversed-phase column (XBridge C18, 3.5 μm, 50 × 2.1 mm; Waters, Waltham, MA) at a flow rate of 500 μL/min (mobile phase A consisted of 100% HPLC grade water with 0.1% formic acid, and mobile phase B contained 100% acetonitrile with 0.1% formic acid). The mass spectrometer was operated under the following conditions: positive ion turbo-ionspray mode, ionspray potential, 5.0 kV; interface temperature, 400°C; curtain gas, 20; CAD gas, 6; GS1, 70; and GS2, 30. The conditions for PHA666859 were as follows: MS/MS transition, m/z 416.16→340.94; declustering potential, 91; and collision energy, 41. The conditions for buspirone (internal standard) were as follows: MS/MS transition, m/z 386.3→122.1; declustering potential, 80; and collision energy, 40. Peak areas were then determined (Analyst software, ver. 1.4.1; ABI). Quantitation was performed by linear regression with a 1/x² weighing. The lower limit of quantitation for the assay was 1 ng/mL. The upper limit of quantitation for the assay was 5000 ng/mL.

**Statistical Analysis**

Data are expressed as the mean ± SD. Statistical analysis was performed with ANOVA, followed by Fisher’s test. *P < 0.05* was considered statistically significant.

**RESULTS**

To select an effective dose and route of administration of PHA666859, we administered the drug in initial studies via gavage (3 mg/kg body weight [BW]/d) or in the diet (at 2.5, 25, or 135 mg/kgBW/d) for 2 months to diabetic animals. Administration of the lowest dose of PHA666859 had no effect on the expression or phosphorylation of p-HSP27 (a downstream biomarker of p38 activity) whether by gavage or in the food and so will not be discussed further.

Phosphorylation of p38 MAPK (p-p38) is known to activate the enzyme, and we therefore measured p-p38 at threonine 180/tyrosine 182. Retinas from control rats having insulin-deficient diabetes that was not treated with the p38 MAPK inhibitor showed a significant increase in p-p38, and administration of PHA666859 at a dose of 25 mg/kgBW/d significantly inhibited this change (Fig. 2a).

Since phosphorylation of HSP27 was known to be dependent on p38 MAPK activity, we measured the ability of the drug to inhibit the diabetes-induced increase in phosphorylation of HSP27 as a biomarker of p38 MAPK activity. Total expression of HSP27 was also measured. Both of the two higher doses of the orally administered drug significantly inhibited phosphorylation of HSP27 (Figs. 2b, 2c). Based on these findings, we...
administered PHA666859 in the diet to diabetic animals for long-term studies at a dose intended to be equivalent to 25 mg/kgBW/d. Although the data indicate that PHA666859 is an effective inhibitor of p38 MAPK activity in vivo, the data demonstrated inhibition of p38 activation as well (Fig. 2a). Although several classes of p38 inhibitors have been shown to block both activation and activity of p38MAPK directly through their interaction with this kinase, the possibility cannot be ruled out that PHA666859 modulates a target up-stream of p38 MAPK in addition to its inhibitory effect on the target.

Diabetic rats in the long-term study were hyperglycemic and failed to gain weight at a normal rate. The severity of hyperglycemia was assessed every 2 to 3 months by determining glycated hemoglobin (GHb). GHb levels in the two diabetic groups (diabetic control and diabetic treated with PHA666859 in the diet) were comparable throughout the duration of the experiment and were significantly greater than normal (Table 1). GHb and random blood glucose values were similar in the 2- and 10-month studies. Over the 10 months of study, the actual intake of PHA666859 was determined to average 27 mg/kgBW/d. Blood levels of drug at this dose remained elevated throughout the day, and averaged 355 ± 88 ng/mL at 8 AM and 266 ± 56 at 4 PM (control animals had undetectable levels in the blood).

The retinal vessels of control rats with 10 months’ duration of diabetes exhibited the expected capillary degeneration and pericyte ghosts compared with the nondiabetic control animals (Fig. 3). Daily consumption of PHA666859 from the onset of diabetes significantly inhibited each of these diabetes-induced lesions.

In an effort to gain insight pertaining to how the beneficial effects of PHA666859 on retinopathy were mediated, we also measured other abnormalities that have been postulated to be important in the pathogenesis of retinopathy. Diabetes caused a significant increase in leukostasis and generation of superoxide (Fig. 4), expression of ICAM-1 and iNOS, nitration of retinal proteins (nitrotyrosine; Fig. 5), and phosphorylation of BAD at serines 112 and 136 and MEK at serines 217/221 (Fig. 6). Oral administration of PHA666859 soon after the onset of diabetes inhibited each of these abnormalities. The effect of drug on the diabetes-induced increase in poly(ADP ribosyl)ation of retinal proteins was mixed, with the inhibitor significantly inhibiting the poly(ADP-ribosyl)ation of retinal protein at approximately 132 kDa (Fig. 7), but this effect was not apparent at other molecular weights (not shown). The p38 MAPK inhibition did
not have an effect on the diabetes-induced upregulation of COX-2 or MAPK-activated protein kinase 2 (not shown).

Beneficial effects of p38 MAPK inhibition in diabetes were not limited to the retina. Diabetes enhanced sensitivity of peripheral nerves to light touch in the rats (tactile allodynia; Fig. 8), and administration of the p38 MAPK inhibitor significantly suppressed this diabetes-induced hypersensitivity of sensory function. The mechanism of this action was not explored further.

**DISCUSSION**

p38 MAPK is one of three major MAPK signaling pathways triggered by a variety of extracellular ligands and stresses, including proinflammatory cytokines, reactive oxygen species, growth factors, osmotic stress, and UV irradiation. p38α is the major isofrom of p38 MAPK associated with inflammation, and inhibition of p38 MAPK activity blocks upregulation of a variety of proinflammatory mediators, including monocyte chemotactant protein-1, granulocyte-macrophage colony-stimulating factor, vascular endothelial growth factor, COX-2, and iNOS, matrix metalloproteinases, leukotrienes, and adhesion molecules required for leukostasis and leukocyte emigration. Likewise, NADPH oxidase and the resulting superoxide generation are under the control of p38 MAPK.

Diabetes has been found to activate p38 MAPK in a variety of tissues, including kidney, nerve, vasculature, and heart. In vitro studies likewise have shown that high glucose can activate a p38 MAPK signaling pathway in aortic, renal (mesangial), and pancreatic β cells.

The present study demonstrates that p38 MAPK activation plays a role in the long-term vascular histopathology of diabetic retinopathy. Inhibition of p38 MAPK activation in diabetes inhibited the diabetes-induced death of endothelial cells and pericytes and significantly inhibited degeneration of retinal capillaries. This finding is important, because capillary nonperfusion and degeneration are believed to play a major role in the eventual development of retina ischemia and subsequent development of retinal neovascularization.
Accumulating evidence suggests that inflammation plays a critical role in the development of the early vascular lesions of diabetic retinopathy. Inhibition of this inflammatory cascade at any of multiple steps can inhibit the histopathology characteristic of early stages of diabetic retinopathy in animals, thus providing strong evidence of an important role of inflammation in the pathogenesis of diabetic retinopathy. p38 MAPK is known to regulate gene expression of inflammatory proteins via effects on multiple transcription factors, but it also is able to regulate NF-kB by controlling the phosphorylation of histone H3 in chromatin at NF-kB-binding sites of inflammatory genes, including IL-8 and MCP-1, and the stimulation of NF-kB DNA protein binding at the promoter for iNOS. Inhibition of p38 was found to inhibit the generation of iNOS and nitrotyrosine and to partially inhibit the death of retinal pigment epithelial cells cultured in high glucose. Our finding that inhibition of p38 MAPK in diabetic animals inhibited the increase in leukostasis and expression of retinal iNOS suggests that p38 MAPK activation in the retina is proinflammatory in diabetes and that inhibition of retinopathy by p38 MAPK inhibition is most likely due in part to inhibition of those inflammatory processes.

The therapy also may act via other mechanisms. p38 MAPK activation is involved in superoxide production, as illustrated by the inhibition of angiotensin II-dependent hypertension, organ damage, and superoxide anion production after inhibition of p38 MAPK. p38 MAPK has been reported to activate NADPH oxidase by enhancing phosphorylation and assembly of NADPH oxidase subunits and inhibition of p38 MAPK inhibits activation of NADPH oxidase. In our study, treatment with a p38 MAPK inhibitor suppressed the diabetes-induced increase in production or accumulation of superoxide by the retina, possibly through direct effects on NADPH oxidase or transcriptional regulation of inflammatory proteins and cytokines.

p38 MAPK lies upstream of HSP27 and activates the heat shock protein via phosphorylation. Inhibition of p38 MAPK in our study inhibited the diabetes-induced increase in phosphorylation of HSP27, and this effect was associated with an inhibition of the development of vascular disease. Phosphorylation of HSP27 has been shown to impair its chaperone function, and a mutant having a tertiary structure that mimics phosphorylated Hsp27 is less able to protect against oxidative stress than is the wild-type molecule. Likewise, phosphorylation of HSP27 is less able to protect against oxidative stress than is the wild-type molecule.49,50 Thus, inhibition of phosphorylation of HSP27 or BAD after inhibition of p38 MAPK may have contributed to the beneficial effects of PHA666859 therapy.

Diabetic rats also develop a hypersensitivity to light touch compared with nondiabetic control animals, similar to what develops in some diabetic patients.51 Inhibition of p38 MAPK has beneficial effects related to nerve function, including regulation of inflammatory heat hyperalgesia and mechanical allodynia.52,53 In other diseases, p38 MAPK is known to play an important role in pain responses mediated via the central nervous system through expression of COX-2 and other mediators.54 The present studies provide evidence that p38 MAPK activation also plays a role in the tactile allodynia that develops in diabetes.

Acknowledgments

The authors thank Terri Quenzer for bioanalytical work.

References


