Phosphodiesterase (PDE) 4 inhibitors are a class of drugs that can provide novel therapies for asthma and chronic obstructive pulmonary disease. Their development is frequently hampered by the induction of vascular toxicity in rat mesenteric tissue during preclinical studies. Whereas these vascular lesions in rats have been well characterized histologically, little is known about their pathogenesis and in turn, sensitive and specific biomarkers for preclinical and clinical monitoring do not exist. In order to investigate the early molecular mechanisms underlying vascular injury, time-course studies were performed by treating rats for 2–24 h with high doses of the PDE4 inhibitor CI-1044. Transcriptomics analyses in mesenteric tissue were performed using oligonucleotide microarray and real-time RT-PCR technologies and compared to histopathological observations. In addition, protein measurements were performed in serum samples to identify soluble biomarkers of vascular injury. Our results indicate that molecular alterations preceded the histological observations of inflammatory and necrotic lesions in mesenteric arteries. Some gene expression changes suggest that the development of the lesions could follow a primary modulation of the vascular tone in response to the pharmacological effect of the compound. Activation of genes coding for pro- and antioxidant enzymes, cytokines, adhesion molecules, and tissue inhibitor of metalloproteinase 1 (TIMP-1) indicates that biomechanical stimuli may contribute to vascular oxidant stress, inflammation, and tissue remodeling. TIMP-1 appeared to be an early and sensitive predictive biomarker of the inflammatory and the tissue remodeling components of PDE4 inhibitor-induced vascular injury.

Key Words: vascular injury; PDE4 inhibitor; gene expression; TIMP-1; rat.

Phosphodiesterase type 4 (PDE4) inhibitors have gained growing interest in the last decade as potential therapies in the treatment of asthma and chronic obstructive pulmonary disease (COPD, Zhang et al., 2005). Indeed, by inhibiting the major cyclic adenosine monophosphate (AMP)–hydrolyzing PDE found in inflammatory and immune cells, this class of compounds possesses both immunomodulatory and anti-inflammatory actions (Barnette, 1999). In addition, PDE4 inhibitors can significantly reduce the bronchoconstriction component of these airway diseases since PDE4 is also present, beside the PDE3 isoenzyme, in airway smooth muscle (Torphy, 1998).

Despite reports of the efficacy of PDE4 inhibitors in some Phase III clinical trials, their development for the treatment of asthma and COPD is hampered by the presence of dose-limiting side effects. In addition to the gastrointestinal side effect of nausea, vomiting, and diarrhea observed in human volunteers, regulatory agencies are particularly concerned by the development of vascular injury in laboratory animals during preclinical studies (Giembycz, 2006; Kerns et al., 2005; Kroegel and Foerster, 2007). Vascular lesions following PDE4 inhibitors administration have mostly been reported in rats and the mesenteric vasculature appears to be one of the most sensitive vascular beds in this species (Daguès et al., 2007; Dietsch et al., 2006; Giembycz, 2006; Larson et al., 1996; Mecklenburg et al., 2006; Slim et al., 2002, 2003). Histopathologically, mesenteric vascular injury is characterized by perivascular edema and mixed inflammatory cell infiltration associated with medial necrosis and hemorrhage. Due to the lack of safety margin, the development of vascular and perivascular lesions following treatment with PDE4 inhibitors has been identified by the Food and Drug Administration as a significant safety issue that requires rigorous monitoring in clinical trials (Giembycz, 2006).

There is currently no sensitive and reliable biomarker for both preclinical and clinical monitoring of the vascular lesions induced by PDE4 inhibitors and their pathogenesis is still unclear. They are thought to result from hemodynamic changes that are produced by excessive and prolonged vasodilatation of specific vascular beds (Giembycz, 2006). Indeed, similar lesions have been reported with many vasodilator compounds of various pharmacological classes in rat, as well as in nonrodent species such as dog and monkey (Albassam et al., 1999; Enerson et al., 2006; Joseph, 2000; Kerns et al., 2005; Losco et al., 2004). In order to better understand the
pathogenesis of the vascular lesions induced by the PDE4 inhibitors, the primary toxicity of this class of compound has recently been investigated. Mecklenburg et al. (2006) have shown that rats treated with the selective PDE4 inhibitor BYK169171 for 1–28 days developed a non purulent mesenteritis, characterized by proliferation of fibroblasts and macrophages, prior to the segmental necrosis of the vessels. We also reported that the inflammatory response preceded the development of fibrinoid necrosis in the mesenteric arteries of rats treated for 1–3 days with the selective PDE4 inhibitor CI-1044 (Daguet et al., 2007). Additionally, we observed that histological observation of mesenteric arterial lesions induced by CI-1044 coincided with altered expression of genes involved in vascular tone regulation, tissue remodeling, and inflammatory and oxidative stress responses.

The objectives of the present studies were (1) to characterize the early gene expression changes that precede the onset of the histopathological changes induced by the PDE4 inhibitor CI-1044 in rat mesentery; (2) to derive from the gene expression analysis the identification of soluble biomarkers that appear with the earliest manifestations of vascular injury. For these purposes, a first time-course study was performed in rats treated with CI-1044 for 2, 4, 8, 16, and 24 h and gene expression changes were analyzed in mesenteric tissue using microarray technology. A second study was conducted in rats treated for 2, 4, 8, 12, and 16 h to confirm the changes observed in the first study using quantitative real-time reverse transcription–PCR (QRT-PCR). This analysis was performed on mesenteric tissue for a selection of early activated genes. In addition, proteins implicated in inflammation or tissue remodeling were measured in blood samples.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats 7 weeks of age were obtained from Charles River Laboratories (St Germain-sur-l’Arbresle, France). They were housed separately in an environmentally controlled room (20°C–22°C, 60 ± 20% relative humidity) with a 12-h light/dark cycle. Animals had free access to food pellet and drinking water. The experiments began after a 2-week acclimation period during which the animals were observed daily to ensure they were in good health. All experiments were performed according to Good Laboratory Practice guidelines and protocols were approved by the laboratory animal ethical committee.

CI-1044 Formulation and Administration

CI-1044 [(R)-N-[9-amino-3,4,6,7-tetrahydro-4-oxo-1-phenyl-pyrrolo[3,2,1-j,k][1,4] benzodiazepin-3-yl]-3-pyr-idinecarboxamide], with an approximate purity of 98.8%, was used in both studies reported here. Dosing solutions were prepared by suspending CI-1044 in methylcellulose as a 0.5% (wt/vol) aqueous solution at concentrations of 8 and 16 mg/ml, for the 24- and the 16-h time-course study, respectively. The formulated materials were stored at ambient temperature, protected from light. Compound or vehicle (methylcellulose only) was administered in one occasion, by esophageal intubation, in a volume of 10 ml/kg.

Study Design

Twenty-Four-Hour Time-Course Study

Rats received CI-1044 dose of 80 mg/kg and were euthanized, by exposure to CO2 gas, 2, 4, 8, 16, or 24 h following the administration. There were six animals for the 2-h time-point and three animals for the following time-points. Additional six or three rats were given the vehicle control for the same time as to the corresponding treated group. A portion of mesenteric tissue was sampled and stored at −80°C until RNA isolation.

Sixteen-Hour Time-Course Study

In order to examine in further details the changes detected in the first 24-h study, a second time-course study was performed. Groups of five animals/sacrifice time-point received a single oral dose of 160 mg/kg and groups of three animals/sacrifice time-point received the vehicle alone. One treated group and one control group were sacrificed 2-, 4-, 8-, 12-, or 16-h postdose. A portion of mesenteric tissue was sampled and stored at −80°C until further processing. Blood samples were obtained by puncture of the thoracic aorta during necropsy. A first sample of blood (3 ml) was collected into a dry tube for serum levels of interleukin 6 (IL6) and tissue inhibitor of metalloproteinase 1 (TIMP-1). A second amount of blood (2.9 ml) was collected into plastic tube containing ethylenediaminetetraacetic acid for multianalyte profiles.

Histopathology

Remaining mesenteric tissues were rinsed with saline solution (NaCl 9 g/l) and fixed by immersion in 10% formalin solution (Lillie). Fixed tissues were embedded in paraffin wax. 4- to 5-µm-thick sections were cut, stained with hemalun and eosin, and examined by light microscopy. The degree of inflammation was determined according to the approximate number of inflammatory cells and extension of the tissue infiltration.

Total RNA Isolation from Rat Mesenteric Tissue

Total RNA was isolated from about 50 mg mesenteric tissues using the Rneasy Mini Kit from Qiagen. The concentration and purity of the RNA were determined by spectrophotometry and their integrity was confirmed using an RNA 6000 Nano Assay kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Berlin, Germany). RNA samples were stored at −80°C until assayed.

Microarray Experiment of the 24-h Time-Course Study’s Samples

Microarray Preparation

The preparation and processing of labeled and fragmented cRNA targets for microarray hybridization was performed according to the manufacturer’s protocols (Affymetrix, Inc., Santa Clara, CA). Briefly, first and second strand complementary DNA (cDNA) were synthesized from 2 µg of total RNA and purified using GeneChip Sample Cleanup Module (Qiagen). Biotin-labeled cRNA was generated from double-stranded cDNA using a BioArray High-Yield RNA transcript-labeling kit (Enzo Biochem, Farmingdale, NY) and was purified using the GeneChip Sample Cleanup Module. At each step, integrity of cDNA and cRNA was checked using Agilent 2100 Bioanalyzer. After the fragmentation step, 15 µg of the biotinylated cRNA was hybridized to the Rat Genome oligonucleotide Array containing ~8800 rat-specific probe sets (RG_U34A; Affymetrix, Inc.) for 16 h at 45°C. The probe arrays were then washed, stained with phycoerythin–streptavidin conjugate (Molecular Probes, Eugene, OR), and scanned with a GeneArray scanner (Agilent).

Bioinformatic Analysis

Scanned images of the arrays were converted to numerical data with the Affymetrix Microarray Suite (MAS 5.0) software and outpotted to tab-delimited text files containing Affymetrix Probe Set ID, signals, present, marginal, or absent detection calls, and detection p values for each feature on the array. The data were imported into the GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA) and analyzed to determine gene expression profiles.
Normalization. Normalization was performed by dividing every measurement of each array by the median of all measurements in that array (per chip normalization). Data were then log transformed for further bioinformatic analysis.

Samples clustering. Unsupervised hierarchical clustering was performed using GeneSpring software to classify mesenteric tissue samples into groups that have similar gene expression pattern. The Pearson correlation was used as a similarity measure, based on gene expression levels, with no gene preselected.

Filtering and selection for differentially expressed genes. To determine whether a specific gene is actually present in a sample, Affymetrix Microarray Suite software categorizes genes into present (P), marginal (M), or absent (A) calls based on the relative signal intensity of the transcripts on the GeneChip. This flag was used in order to filter out probe sets that resulted in absent calls in any of the samples. The next step of analysis consisted in the identification of the differentially expressed probe sets across the groups of samples under-scored by hierarchical clustering. As previously described, statistical power was increased by comparing the animals with vascular lesions to unaffected animals instead of comparing untreated and treated animals at different time-points (Daguès et al., 2007). Thus, Welch approximate t-test was applied for each gene and the subsets of genes with an adjusted p value less than 0.05 were selected as significantly differentially expressed genes. p values were corrected to reduce false positive using Benjamini and Hochberg (1995) method for estimating the false discovery rate. Genes that were either increased or decreased more than twofold (fold change ≥ 2 or ≤ 0.5) between the groups of samples were then selected and functionally categorized. In addition, in order to focus on early molecular changes, i.e., before the manifestation of histopathological changes, we observed the expression profile of the differentially expressed probe sets in treated samples at each time-point. A gene was considered differentially expressed in treated group compared to control group when its expression level changed by at least twofold.

Reverse Transcription and Real-Time PCR of the 16-h Time-Course Study’s Samples

Two micrograms of each total RNA sample was used to generate cDNA using the High Capacity cDNA Archive Kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Based on the gene expression changes highlighted by microarray experiments, PCR assays were performed to measure the expression levels of 23 genes with primers and TaqMan probes designed by the company (Applied Biosystems). The probe contained a minor groove binder and nonfluorescent quencher at the 3’ end. 18S ribosomal RNA (rRNA) expression levels were used for normalization. Quantitative real-time PCR was performed using either rat sequence-based TaqMan Gene Expression assays or TaqMan Low Density Arrays (TLDA). Both systems use the same standard conditions determined by Applied Biosystems and reactions are performed with an ABI Prism 7900 Sequence Detection System. For the TaqMan Gene Expression Assays, PCR amplification was performed in duplicate in a 25-μl reaction volume consisting of cDNA corresponding to 50 ng total RNA, 1 × TaqMan Universal Master Mix and 1 × primers and probe solution. For the TLDA, the primers and probe sets were preloaded in triplicate into each of the 384 wells of the array and 100-μl reaction volume consisting of cDNA corresponding to 100 ng total RNA combined with 1 × TaqMan Universal Master Mix was loaded into each array port. According to the manufacturer’s guidelines, the level of expression was calculated based upon the PCR cycle number (Ct) at which the exponential growth in fluorescence from the probe passes a certain threshold value. For each sample, relative gene expression level was determined by subtracting the Ct value of the housekeeping gene 18S rRNA to the Ct value of the target gene (ΔCt = CtTarget gene − Ct18S rRNA). Relative quantification (fold change) between different samples (e.g., treated vs. control) was then determined according to the 2−ΔΔCt method (Livak and Schmittgen, 2001) (ΔΔCt = ΔCt treated sample − ΔCt control sample).

QRT-PCR was performed with samples of both the 24- and the 16-h time-course studies. The results were similar for the selected genes and we only report here the most complete set of data obtained with the 16-h time-course study.

Measurement of Plasma/Serum Proteins in the 16-h Time-Course Study’s Samples

Plasma samples collected from the 16-h time-course study were evaluated for multianalyte profiles (Rules Based Medecine, Inc., Austin, TX). Using this system, plasma samples were evaluated for 60 different plasma antigens that included cytokines, growth factors, acute phase proteins, and other biomarkers of inflammation.

In addition, plasma levels of interleukin (IL) 6 and serum level of tissue inhibitor of metalloproteinase (TIMP)-1 were determined using commercially available enzyme-linked immunoadsorbent (ELISA) kits, according to the manufacturers’ instructions (R&D Systems, Minneapolis, MN). The lower limits of detection for these ELISAs were 21 and 3.5 pg/ml for IL6 and TIMP-1, respectively. The plates were read at 450 nm and the absorbances were transformed to pg/ml using standard curves prepared with IL6 and TIMP-1 standards provided with the kits.

Statistical Analysis

Statistical analysis of both QRT-PCR data and protein measurements were performed using SAS/STAT software (SAS OnlineDoc, Version 8, Cary, NC: SAS Institute, Inc., 1999).

QRT-PCR Data

For every time-point, individual fold changes (FC) were calculated, for each gene, by associating the ΔCt of each treated animal to the ΔCt of each control animal. Since there were three control animals and five treated animals per time-point, 15 fold changes were thus calculated for every time-point to obtain a geometric mean FC ± geometric standard deviation. The statistical analysis was performed on the log transformation of the individual fold change values. For treated groups of each time-point, one-tailed tests were used for every upregulated gene to test that the fold change value was statistically significantly greater than 2. These tests were performed at the 5% significance level.

Protein Measurements

For the multianalyte profiles, only proteins showing 50% of valid data (i.e., nonmissing values or values upper the limit of detection) and with at least three measurements recorded for each treated group were submitted to the statistical analysis. For each protein (including IL6 and TIMP-1), normality was tested using a Shapiro–Wilk test and a Levene test was used to check variance homogeneity among the groups. A two-factor analysis of variance was then performed to test for treatment and time effect. When there was a significant treatment × time interaction, control and treated groups were compared at each time-point. The SAS Univariate and Generalized Linear Model procedures were used to perform the analyses. All tests were two-tailed tests performed at the 5% significance level.

RESULTS

Histopathology

Treatment-related microscopic findings were observed in the mesentery in both the 16-h and the 24-h time-course studies. At 16-h postdose, the lesions were characterized by minimal to mild inflammation consisting of interstitial edema with focal/multifocal perivascular to interstitial inflammatory infiltrates.
Infiltrates were composed mainly of neutrophils. At 24-h postdose, inflammatory findings were variably accompanied by focal/multifocal vascular fibrinoid necrosis. Overall, morphological findings in the rat mesentery were similar to those observed in previously published histopathologic examinations (Daguès et al., 2007).

Samples Clustering

As shown in Figure 1, with the exception of one animal (sample 129, treated 24 h), the five animals that showed inflammation and/or necrosis by histopathological examination (three treated animals for 16 h and two treated animals for 24 h) clustered together (Cluster 1) while all the animals with no lesion (treated or untreated) gathered together (Cluster 2). Apart from these two main clusters, there were three outlier samples (27 [control, 24 h], 105, and 106 [treated, 2 h]) for which retrospective quality control issues with the preparation and processing were detected. These three outliers as well as sample 129 were discarded for subsequent filtering analysis to avoid introducing a bias in the selection of the genes specifically affected by CI-1044.

Selection of Differentially Expressed Genes

A subsequent statistical analysis was applied to select the genes differentially regulated between Clusters 1 and 2 (summarized in Fig. 2). After normalization and hierarchical clustering, 58.5% of the probe sets (5313) passed the filtration based on flags present or marginal in at least one of all the sample arrays. Using Welch approximate t-test, 683 probe sets were found to be significantly differentially regulated between the two main groups of samples clustered by the hierarchical clustering analysis. Of these, the expression of 116 probe sets increased and the expression of 79 probe sets decreased by at least twofold in groups 1 compared to group 2. After excluding all expressed sequence tags (ESTs), 95 upregulated probe sets and 68 downregulated probe sets were further categorized in
gene groups according to their corresponding biological function (Fig. 3). Since one gene can be represented by several probe sets, there were actually 74 upregulated genes and 49 downregulated genes. A majority of the upregulated genes coded for proteins associated with stress responses (inflammation, oxidative stress), metabolism, and signal transduction. The downregulated genes coded mainly for enzymes implicated in the metabolism of lipid and glucose and for proteins involved in signal transduction and transport. In addition, analysis of gene expression changes in treated samples at each time-point allowed us to highlight a certain number of genes whose expression was altered earlier than the detection of the lesions by histopathology (Table 1). This was particularly the case for upregulated genes whose expression level changes could be detected as soon as 2-h postdose. Based on these observations and in order to focus on early molecular changes, real-time PCR was performed on a subset of upregulated genes in the second time-course study.

QRT-PCR Analysis of Expression Profiles of Specific Transcripts

The relative expression levels of 21 out of 23 genes representative of the major functional categories eluded with the microarray analyses are shown in Table 2. Gene expression level of PDE4A and nitric oxide synthase (NOS) 2 are not shown in the table as they were not detectable in any of the samples. In the category defense/stress/inflammation responses, the genes coding for IL6 and metallothionein 1a (Mt1a) were significantly upregulated 2-h postdose by up to 15- and 17-fold, respectively. Their expression was also increased 12- and 16-h postdose for IL6 (up to 59 fold) and 4-, 12-, and 16-h postdose for Mt1a (up to 17-fold). In addition, intercellular calcium binding protein (MRP8) and intracellular calcium binding protein (MRP14) showed similar gene expression profiles and were significantly upregulated from 4-h postdose (up to ninefold), and these increases were also consistent (fold change > 2) at the 2-h time-point. In the extracellular matrix category, gene expression level of TIMP-1 was statistically significantly increased at both 4- and 16-h time-point (up to sixfold). In the blood coagulation and proteolysis categories, statistically significant time-dependent increase in fibrinogen was observed from 8-h postdose, whereas genes coding for the plasminogen activator, tissue (tPA), and its inhibitor, the plasminogen activator inhibitor type 1 (PAI-1) were increased at both 2- and 16-h postdose (up to 20-fold) and at 4-h postdose (fivefold), respectively. In metabolism and signal transduction categories, apart from the genes coding for NOS 3 and PDE4B whose expression was unchanged, all the others genes in these categories were statistically significantly increased 4-h postdose.

Quantification of Circulating Proteins in Rat Blood

Due to a lack of blood samples left, TIMP-1 was not measured in two serum samples (from animal 6, control 4 h and animal 105, treated 2 h) and plasma IL6 quantification and multianalytes analysis were not performed in two plasma samples (from animal 1, control 2 h and animal 119, treated 12 h). In addition to TIMP-1 and IL6, statistical analysis of the multianalytes data showed significant treatment-related effects for three out of 60 proteins, namely fibrinogen, C-reactive protein (CRP), and haptoglobin (Table 3). TIMP-1 and CRP concentrations were statistically significantly increased from 8-h postdose. Of note, among the five proteins represented in this table, TIMP-1 showed the highest amplitude of changes. Indeed, its concentration increased, in a time-related manner, by 29% at the 4-h time-point and reached 176% 16-h postdose. Fibrinogen concentration was statistically significantly increased from 12-h postdose. IL6 was detected only in treated group and, in turn, statistical analysis of its induction was not possible. The high values of the standard deviation indicated an interindividual variability. However, IL6 concentration was considered consistently increased at the 16-h time-point. Haptoglobin was mildly increased from 8-h postdose, though not statistically significantly. Overall, based on the amplitude and the time of appearance of the changes in this study, TIMP-1 appeared to be the earliest and most sensitive candidate marker of CI-1044 effect.

DISCUSSION

Vascular injury following administration of PDE4 inhibitors in rats is characterized histologically by primary inflammatory cells infiltration followed by necrosis and hemorrhage of the vascular wall (Daguès et al., 2007; Dietsch et al., 2006;
Mecklenburg et al., 2006). It has been argued that these lesions occur in vascular beds susceptible to the development of spontaneous idiopathic vascular diseases. The mechanisms for this selectivity are unclear but they may be related to localized changes in blood flow (Kerns et al., 2005). We observed previously that the severity of the mesenteric arterial lesions in rat following a treatment for 1, 2, or 3 days with the PDE4 inhibitor CI-1044 coincided with altered expression of genes involved in inflammatory and oxidative stress responses, vascular tone regulation, and tissue remodeling (Daguès et al., 2007). It was hypothesized that the vascular lesions observed in rat mesenteric arteries could be the result of hemodynamic forces such as altered shear stress possibly linked to an exaggerated pharmacological effect of the compound (Daguès et al., 2007; Kerns et al., 2005). Supporting evidence for this hypothesis was demonstrated in the present studies by the early expression changes of genes implicated in the vascular tone regulation. Induction of both prostacyclin synthase, which mediates vasodilatory properties, and of endothelin receptor type B, which is one of the two receptors for the vasoconstrictor protein endothelin-1, was observed at the 4-h time-point. These changes are indicative of a modulation of the vascular tone and suggest an ischemia/reperfusion-like response. The initial event of this would be a vasodilation induced by the increase in cyclic AMP (cAMP) levels following PDE4 inhibition by CI-1044. Increase in cAMP levels is exemplified by the identification, among the genes upregulated from the 2-h time-point, of cAMP-responsive element modulator, a transcription factor activated by the cAMP-dependent protein kinase A (De Cesare and Sassone-Corsi, 2000). This vasodilation would then be compensated by a constricting response, leading to transient ischemia, which was translated in the present studies by the increased messenger

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Probe Sets that were Up- or Downregulated in Rat Mesenteric Tissue According to Treatment Duration with CI-1044</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of probe sets altered according to treatment duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
</tr>
<tr>
<td>Upregulated</td>
</tr>
<tr>
<td>Downregulated</td>
</tr>
</tbody>
</table>

Note. Data represent the number of probe sets, among the 95 upregulated and the 68 downregulated probe sets highlighted by the filtering analysis, whose expression level changed by twofold or more between treated and control samples at each treatment time. ESTs have been dismissed from this table.
RNA (mRNA) expression 4-h postdose of some PDE3 and four isoforms known for decreasing cAMP levels in vascular smooth muscle cells (VSMCs) (Torphy, 1998). The first molecular events following this ischemic and subsequent reperfusion-like response might be the increased generation of reactive oxygen species (ROS) and the release of proinflammatory mediators as these mechanisms have been proposed in the initiation of the damaging processes in the context of ischemia/reperfusion (Kumar et al., 2005). In our studies, early production of ROS was suggested in the mesenteric vasculature through the mRNA increase of the pro-oxidant enzyme xanthine dehydrogenase at the 4-h time-point. Xanthine oxidase, which is posttranslationally generated from xanthine dehydrogenase, is an important source of ROS and has been implicated in various clinicopathologic entities, including ischemia/reperfusion injury, and multisystem organ failure (Meneshian and Bulkley, 2002). In contrast, the simultaneous increases of the antioxidant enzymes glutathione peroxidase 3 and superoxide dismutase 2 (Faraci and Didion, 2004; Loscalzo et al., 2005) in our studies suggested an imbalance in ROS production. ROS, which can be produced by migrating inflammatory cells, endothelial cells (ECs), VSMCs, and fibroblasts, could play a central role in the development of vascular injury through their participation in remodeling the vessel wall (Fortuño et al., 2005; Li and Shah, 2004; Taniyama and Griendling, 2003). In support of a role for oxidative stress, Slim et al. (2003) have shown increased inducible NOS2 expression and nitrotyrosine immunoreactivity in mesenteric arteries exhibiting significant inflammatory infiltrates after 4-days treatment with the PDE4 inhibitor CI-1018.

In response to shear and oxidative stress, many functions of the endothelium can be altered reducing its capacity to

### TABLE 2

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene description</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defense/stress/inflammation responses</td>
<td>Interleukin-6</td>
<td>15.38 ± 10.47*</td>
<td>1.55 ± 6.33</td>
<td>1.43 ± 20.29</td>
<td>7.24 ± 2.62**</td>
<td>59.31 ± 3.00***</td>
</tr>
<tr>
<td></td>
<td>Interacellular calcium-binding protein (MRP8)</td>
<td>2.30 ± 2.36</td>
<td>5.12 ± 1.34***</td>
<td>7.87 ± 2.95***</td>
<td>4.75 ± 1.90**</td>
<td>9.46 ± 1.56***</td>
</tr>
<tr>
<td></td>
<td>Intracellular calcium-binding protein (MRP14)</td>
<td>2.21 ± 2.34</td>
<td>5.28 ± 1.42***</td>
<td>8.25 ± 4.72***</td>
<td>4.43 ± 1.92**</td>
<td>6.35 ± 1.67***</td>
</tr>
<tr>
<td></td>
<td>Selectin, platelet</td>
<td>1.70 ± 1.49</td>
<td>3.37 ± 1.73*</td>
<td>4.55 ± 3.63</td>
<td>1.05 ± 1.41</td>
<td>3.07 ± 2.96</td>
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<tr>
<td></td>
<td>Metallothionein 1a</td>
<td>17.32 ± 3.11***</td>
<td>8.38 ± 1.66***</td>
<td>1.22 ± 8.19</td>
<td>16.90 ± 1.74***</td>
<td>11.17 ± 2.26***</td>
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<td>Superoxide dismutase 2, mitochondrial</td>
<td>1.21 ± 1.29</td>
<td>1.77 ± 1.47</td>
<td>2.67 ± 1.86</td>
<td>1.16 ± 1.30</td>
<td>6.14 ± 1.60***</td>
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<tr>
<td></td>
<td>Heme oxygenase 1</td>
<td>1.69 ± 1.27</td>
<td>1.23 ± 1.88</td>
<td>2.75 ± 1.97*</td>
<td>1.45 ± 1.27</td>
<td>2.40 ± 1.49</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>Tissue inhibitor of metallocproteinase 1</td>
<td>1.83 ± 1.25</td>
<td>3.53 ± 1.91***</td>
<td>2.00 ± 1.53</td>
<td>1.69 ± 1.56</td>
<td>5.93 ± 1.63***</td>
</tr>
<tr>
<td>Blood coagulation</td>
<td>Fibrinogen γ</td>
<td>0.61 ± 4.99</td>
<td>1.88 ± 1.99</td>
<td>19.96 ± 4.62**</td>
<td>37.26 ± 3.26***</td>
<td>64.37 ± 3.58***</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activator inhibitor type 1</td>
<td>4.68 ± 2.21**</td>
<td>1.13 ± 1.49</td>
<td>1.16 ± 4.05</td>
<td>1.42 ± 1.19</td>
<td>19.97 ± 2.35***</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>Plasminogen activator, tissue</td>
<td>1.97 ± 1.27</td>
<td>4.70 ± 2.30***</td>
<td>2.61 ± 2.19</td>
<td>1.11 ± 1.33</td>
<td>1.52 ± 1.30</td>
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<td>Metabolism</td>
<td>Prostaglandin I2</td>
<td>1.58 ± 1.48</td>
<td>5.62 ± 2.84***</td>
<td>1.59 ± 1.62</td>
<td>1.27 ± 1.71</td>
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</tr>
<tr>
<td></td>
<td>(prostacyclin) synthase</td>
<td></td>
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<td></td>
<td>Glutathione peroxydase 3</td>
<td>1.44 ± 1.24</td>
<td>3.58 ± 1.80***</td>
<td>1.56 ± 1.63</td>
<td>1.24 ± 1.56</td>
<td>1.61 ± 1.47</td>
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<tr>
<td></td>
<td>Xanthine dehydrogenase</td>
<td>1.69 ± 1.31</td>
<td>4.55 ± 1.96***</td>
<td>2.13 ± 1.72</td>
<td>1.23 ± 1.40</td>
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<td>Phosphodiesterase 3A</td>
<td>1.57 ± 1.35</td>
<td>3.88 ± 2.14***</td>
<td>1.57 ± 1.89</td>
<td>0.87 ± 1.66</td>
<td>0.86 ± 1.28</td>
</tr>
<tr>
<td></td>
<td>Phosphodiesterase 3B</td>
<td>1.36 ± 1.38</td>
<td>2.68 ± 1.57**</td>
<td>1.60 ± 1.29</td>
<td>1.96 ± 1.24</td>
<td>1.79 ± 1.40</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide synthase 3</td>
<td>1.45 ± 1.36</td>
<td>1.95 ± 2.18</td>
<td>1.61 ± 1.64</td>
<td>1.01 ± 1.49</td>
<td>1.96 ± 1.53</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>Phosphodiesterase 4B</td>
<td>1.96 ± 2.59</td>
<td>0.66 ± 1.58</td>
<td>0.50 ± 1.39</td>
<td>0.91 ± 2.08</td>
<td>0.53 ± 3.18</td>
</tr>
<tr>
<td></td>
<td>Phosphodiesterase 4C</td>
<td>1.35 ± 1.16</td>
<td>3.07 ± 2.76**</td>
<td>2.18 ± 1.75</td>
<td>0.51 ± 1.32</td>
<td>1.11 ± 1.25</td>
</tr>
<tr>
<td></td>
<td>Phosphodiesterase 4D</td>
<td>1.52 ± 1.48</td>
<td>3.29 ± 1.72**</td>
<td>1.28 ± 1.82</td>
<td>0.95 ± 1.38</td>
<td>0.76 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>Endothelin receptor type b</td>
<td>1.89 ± 1.23</td>
<td>2.48 ± 1.65*</td>
<td>1.82 ± 2.15</td>
<td>1.26 ± 1.38</td>
<td>1.67 ± 1.23</td>
</tr>
</tbody>
</table>

**Note.** Genes were categorized according to their biological function and fold changes, compared to control, were calculated at each time-point. The relative levels of gene expression were normalized to the 18S rRNA gene. Values are expressed as geometric mean FC ± geometric SD. FC significantly greater than 2: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Concentration of a Selection of Proteins in Blood Samples from Control and CI-1044-Treated Rats

<table>
<thead>
<tr>
<th>Protein</th>
<th>Animal group</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1 (pg/ml)</td>
<td>Control</td>
<td>10,710.3 ± 402.9</td>
<td>10,458.0 ± 285.7</td>
<td>8834.0 ± 416.8</td>
<td>7684.7 ± 784.0</td>
<td>6986.7 ± 1208.2</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>8974.0 ± 612.0</td>
<td>13,497.8 ± 1729.1</td>
<td>16,524.4 ± 1397.0**</td>
<td>15,134.2 ± 4371.9**</td>
<td>19,303.8 ± 6236.2***</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td>−16%</td>
<td>+29%</td>
<td>+87%</td>
<td>+98%</td>
<td>+176%</td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>Control</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>60.6 ± 40.2</td>
<td>72.7 ± 54.6</td>
<td>67.7 ± 35.0</td>
<td>58.3 ± 36.3</td>
<td>158.9 ± 47.7</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>Control</td>
<td>834.5 ± 91.2</td>
<td>833.7 ± 38.0</td>
<td>787.3 ± 107.0</td>
<td>834.7 ± 118.6</td>
<td>897.3 ± 51.4</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>831.2 ± 123.9</td>
<td>926.2 ± 68.0</td>
<td>1083.4 ± 97.4**</td>
<td>1070.8 ± 193.4*</td>
<td>1260.0 ± 115.8***</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td>−0.4%</td>
<td>+11%</td>
<td>+38%</td>
<td>+28%</td>
<td>+40%</td>
</tr>
<tr>
<td>Fibrinogen (µg/ml)</td>
<td>Control</td>
<td>313.5 ± 26.2</td>
<td>395.0 ± 350.8</td>
<td>332.0 ± 118.9</td>
<td>263.7 ± 173.4</td>
<td>300.7 ± 99.4</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>362.0 ± 194.6</td>
<td>365.4 ± 103.1</td>
<td>459.0 ± 39.3</td>
<td>418.8 ± 58.0</td>
<td>512.8 ± 64.6</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td>+15%</td>
<td>+6%</td>
<td>+38%</td>
<td>+59%</td>
<td>+71%</td>
</tr>
</tbody>
</table>

Note: BLQ, below limit of quantification (when individual values were not measurable on the standard curve and/or were below the sensitivity threshold of the Elisa kit (3.5 and 21 pg/ml for TIMP-1 and IL6, respectively; 12, 0.53, and 0.64 µg/ml for fibrinogen, CRP and haptoglobin, respectively) for at least two animals out of three or three animals out of five for the control and the treated groups, respectively); NA, not applicable. Values of concentration are expressed as mean ± SD. Treated versus control groups comparison: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. The percentage of change in treated group versus control group [(mean concentration<sub>treated group</sub> − mean concentration<sub>Control group</sub>)/mean concentration<sub>Control group</sub>] is also indicated.
and TIMPs play an important role in normal tissue homeostasis, but imbalance between the proteolytic enzymes and their tissue inhibitors is thought to be a critical factor in regulating tissue remodeling under pathological conditions such as wound healing/scar formation, angiogenesis, and cancer metastasis. In our studies, whereas no gene expression change of MMPs was observed in the mesenteric vasculature at the time-points evaluated, increase in TIMP-1 mRNA could be detected as soon as 4 h after CI-1044 treatment. Additionally, its increased expression 16-h postdose is consistent with previous toxicogenomics data and showed that TIMP-1 mRNA expression increased with the severity of the mesenteric vascular lesions (Daguès et al., 2007). At protein level, ELISA analysis revealed that TIMP-1 was consistently increased from 4-h postdose in a time-dependent manner and showed the highest amplitude of changes in treated rats compared to controls among all the analytes evaluated. In addition to its early detection in peripheral blood, a dose–response relationship between level of circulating TIMP-1 and the degree of the lesion was also demonstrated with animals treated up to 48 h with CI-1044 (unpublished data).

Similar increases in TIMP-1 have been demonstrated in various rat models of ischemia. In lung, increased TIMP-1 gene expression was observed, together with increased MMP-9 activity and gene expression, 24 h after the induction of ischemia/reperfusion injury (Yano et al., 2001). Wang et al. (1998a) also observed TIMP-1 mRNA induction in a rat model of ischemia-induced brain injury. A potential role of TIMP-1 in brain ischemic tolerance was proposed by the authors based on its increased mRNA expression following a short duration of ischemia, known to result in a subsequent resistance to severe ischemic tissue injury (Wang et al. 1998b). Moreover, increased TIMP-1 protein levels in blood were also described in patients with cardiovascular disorders such as myocardial infarction (Cavusoglu et al., 2006) or inflammatory diseases such as sepsis (Hoffmann et al., 2006) or some type of vasculitis (Bjerckel et al., 2004; Chua et al., 2003). Taken together, this indicates that overexpression of TIMP-1 is associated with inflammation and vascular remodeling. These processes being the main characteristics of vascular lesions induced by PDE4 inhibitors in rats, we propose that TIMP-1 is potentially an early and sensitive biomarker of the inflammatory and the tissue remodeling components of drug-induced vascular injury.

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REFERENCES


