Effects of Modulating In Vivo Nitric Oxide Production on the Incidence and Severity of PDE4 Inhibitor–Induced Vascular Injury in Sprague-Dawley Rats

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Drug-induced vascular injury (DIVI) is observed in rat mesenteric arterioles in response to treatment with phosphodiesterase-4 inhibitors (PDE4i). However, the mechanisms responsible for causing the characteristic vascular lesions are unclear. Nitrotyrosine (NT) adducts, markers of local nitric oxide (NO) production, have been observed in close proximity to the arterial lesions and in the inflammatory cells associated with DIVI. To determine if NO has a direct role in DIVI, rats were treated with the PDE4i CI-1044 at 10, 20, or 40 mg/kg alone or in combination with the nitric oxide synthase inhibitor L-NAME (60 mg/kg) or the nitric oxide donor SIN-1 (30 mg/kg). Mesenteries were collected and processed for microscopic evaluation. NT formation was evaluated in situ via immunohistochemical staining. Serum nitrite (SN), a marker of in vivo NO production, was measured. Compared with vehicle controls, treatment with CI-1044 alone resulted in dose-related increases in the frequency and severity of vascular injury, SN levels, and NT residues. SIN-1 coadministration caused vascular injury to occur at lower doses of CI-1044, compared with CI-1044 alone, with the overall incidence and severity of injury being greater across all CI-1044–dose groups. Following administration of 20 or 40 mg/kg CI-1044, there were also increases in NT immunoreactivity when SIN-1 was coadministered and significant increases in SN. Conversely, coadministration of L-NAME resulted in marked reduction of injury, NT, and SN when compared with CI-1044 alone. The present study suggests that NO production is closely linked to PDE4i-induced vascular injury.

Key Words: drug-induced vascular injury; mesenteric artery; PDE4 inhibitor; nitric oxide; serum nitrite; nitrotyrosine.

Inhibition of the cyclic nucleotide phosphodiesterase-4 (PDE4) enzyme has proven effective at suppressing proinflammatory cytokine production (Burnouf et al., 2000) and relaxes respiratory smooth muscle (Heaslip et al., 1994). These findings suggest that PDE4 is a potential molecular target for treating pulmonary diseases with both inflammatory and constrictive components such as chronic obstructive pulmonary disorder and asthma. The development of PDE4 inhibitors as potential drugs, however, has been limited due to the frequent occurrence of drug-induced vascular injury (DIVI) during preclinical toxicity testing. The published studies on PDE4 inhibitors have been primarily descriptive and provide little information concerning the mechanism by which administration of PDE4i leads to DIVI. This mechanistic uncertainty coupled with the current lack of sensitive and specific noninvasive biomarkers for DIVI presents major obstacles for further clinical development of PDE4 inhibitors.

The mechanisms that drive PDE4 inhibitor-induced vascular injury have not been fully elucidated. Previous studies have examined the light, electron microscopic, immunohistochemical, and genomic aspects associated with DIVI (Dalmas et al., 2008; Zhang et al., 2002). The histopathological changes induced by PDE4 inhibitors have been characterized in several species used for preclinical testing, including rats, dogs, and monkeys (Hanton et al., 2008; Heaslip et al., 1994; Losco et al., 2004; Zhang et al., 2008). Although the reasons are unclear, species differences exist with regard to the localization of the vascular injury, the mesenteric arterial beds seem to be especially sensitive in the rat and cardiac arterioles are more sensitive in the dog and primate (Kerns et al., 2005). In general, histopathological findings are similar in the different vascular beds and include early perivascular edema and inflammation followed by hemorrhage and necrosis of the artery and arteriolar media. Microscopic findings also include arterial endothelial cell (EC) activation as well as multifocal loss of ECs.

The primary effect of inhibition of cyclic nucleotide phosphodiesterase-4 is the elevation in intracellular levels of cAMP (Polson and Strada, 1996). This can have many effects depending on the signaling pathways available in the treated cell.
type. A major component of regulation of vascular tone is nitric oxide (NO). This can be produced in ECs by endothelial nitric oxide synthase (eNOS), which is at the intersection of multiple signaling pathways (Sessa, 2004). One of these pathways is through the cAMP activation of protein kinase A (PKA). Several observations suggest the possibility for a role for NO in association with the vascular injury associated with PDE4 inhibitors. A review of the literature suggests that nitrotyrosine (NT) adducts are consistently colocated with PDE4 inhibitor-induced vascular lesions (Slim et al., 2003; Zhang et al., 2008), and NT residues are formed under certain cellular conditions secondary to NO production. In addition, elevations of serum NO have been observed following PDE4i treatment (Weaver et al., 2008, 2010). Moreover, NO concentrations can have a variety of negative effects on various cell types and on mitochondrial function (Denicola and Radi, 2005; Pacher and Szabo, 2006). NO is the major vasorelaxing factor that is released from the vascular endothelium, but NO also plays a complex role in inflammatory cells. In early inflammation, NO can be proinflammatory, where in late inflammation it may be anti-inflammatory (Fortin et al., 2010). Interestingly, a recent study showed that treatment with CI-1044 resulted in significant vasodilation of the mesenteric arteries in rats (Korkmaz et al., 2009), which were also affected by perivascular and interstitial inflammation.

The primary objective of this study was to examine the effect of modulation of nitric oxide levels in the pathogenesis of CI-1044–induced vascular injury. To achieve this goal, we utilized a nitric oxide synthase (NOS) inhibitor and an NO donor molecule to effectively decrease or increase in vivo NO production. We assessed changes in vascular lesions associated with this modulation and examined markers of NO production, such as nitrotyrosine adducts and serum nitrite (SN).

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley (SD) rats (10–12 weeks old) were obtained from Harlan Laboratories (Frederick, MD) and housed separately in an environmentally controlled room (18–21°C, 40–70% relative humidity) with a 12-h light/dark cycle. Rats were fed certified Purina Rodent Chow #5002 (Ralston Purina Co., St Louis, MO) and had access to water *ad libitum*. The rats were acclimated for at least 1 week prior to experiments and were randomly assigned to different treatment groups (five rats per treatment group). The rats were acclimated for at least 1 week prior to experiments and were randomly assigned to different treatment groups (five rats per treatment group).

The experimental protocol was approved by the Institutional Animal Care and Use Committee, Center for Drug Evaluation and Research, Food and Drug Administration and conducted in an AAALAC-accredited facility.

**Chemicals and Solutions.** CI-1044 [1-(4-morpholinyl)-3-(4-pyridinyl)-2-hydroxy-5-(3-aminopropyl)-2,5-dihydro-1H-pyridin-3-one], a selective inhibitor of PDE4, was obtained from Pfizer (Groton, CT). CI-1044 was suspended in methylcellulose as a 0.5% (wt/vol) aqueous solution and was administered by oral gavage in a volume of 5 ml/kg. Control rats received the same volume of methylcellulose solution.

The NOS inhibitor N\textsuperscript{2}-[iminonoritroamine]methyl]-L-ornihine, methyl ester, monohydrochloride (L-NAME) as well as NO donor molecule N-thiocarbonyl-3-(4-morpholino)-sytndione imine (Molsidomine/SIN-1) were obtained from Sigma (St Louis, MO) and was dissolved in saline for oral gavage dosing. Rats that received neither the NOS inhibitor nor the NO donor were given the same volume of saline. The selective iNOS inhibitor 2-mercaptoethyl)-guanidine sulfate was dissolved in saline for oral gavage dosing.

**Study Design.** The present study consisted of four separate experiments.

**Experiment 1 (CI-1044 and saline).** Rats were dosed by oral gavage with CI-1044 or methylcellulose vehicle daily for 3 days. Rats were concurrently dosed with saline (0.5 ml) by oral gavage once a day for 3 days in place of a nitric oxide modulator (e.g., L-NAME or SIN-1).

**Experiment 2 (CI-1044 and SIN-1).** Rats were dosed by oral gavage with CI-1044 or methylcellulose vehicle daily for 3 days. Rats were concurrently dosed with SIN-1 (0.5 ml) by oral gavage once a day for 3 days.

**Experiment 3 (CI-1044 and L-NAME).** Rats were dosed by oral gavage with CI-1044 or methylcellulose vehicle daily for 3 days. Rats were concurrently dosed with L-NAME (0.5 ml) by oral gavage once a day for 3 days.

**Experiment 4 (CI-1044 and MEG).** Rats were dosed by oral gavage with CI-1044 or methylcellulose vehicle daily for 3 days. A group of rats was concurrently dosed with CI-1044 and MEG (10 mg/kg) by oral gavage twice a day for 3 days.

For all experiments, the rats were euthanized by isofluorane anesthesia followed by exsanguination 24 h after the last dose. Blood was collected from the posterior vena cava for the preparation of serum samples, and the mesentery from each rat was collected as described below.

**Histopathology.** The entire mesenteric arcade, with gastrointestinal tract attached, was collected from each animal at necropsy. Each mesentery was then trimmed to remove the gastrointestinal tract and mesenteric lymph nodes, rinsed with saline (NaCl 0.9%), rolled onto the plunger of a 1-cc syringe, and placed into a small round tissue cassette. The mesentery rolls were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopic evaluation. One section of the mesentery from each animal was examined and each microscopic change was numerically scored from 1 to 4 based on the following criteria:

- **Minimal.** Focal or multifocal lesions that affect a small portion of the mesentery, adventitia, or vessel wall and do not expand or obscure the mesentery, adventitia, or disrupt the vessel wall. In addition, these lesions affect < 10% of the vessels.
- **Mild.** Lesions are multifocal and more extensive affecting up to 25% of the vessels, but the vessel wall remains intact.
- **Moderate.** Coalescing lesions affecting up to 50% of the vessels. Lesions may be transmural and affect the entire circumference of the vessel. In addition, the mesentery or adventitia is expanded and/or obscured.
- **Marked.** Lesions are more widespread affecting more than 50% of the vessels and are more severe with destruction of the vessel wall and extensive mesenteric/adventitial hemorrhage and inflammation.

**Determination of Serum Nitrite Concentrations.** Previous studies have shown that SN levels are highly reflective of the amount of NO formed in vivo (Kelm et al., 1999). In the present study, these measurements were performed using the Total Nitric Oxide Assay (R&amp;D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. Briefly, serum samples were filtered using 10,000 molecular weight cut-off centrifugal filters and reacted with NADH and nitrate reductase enzyme for 30 min at 37°C. Next, Griess reagents were added to the samples and incubated at room temperature for 10 min. The resulting chromophore was read in a spectrophotometer at 540 nm. The samples were run in duplicates with blank and standards. Concentrations of nitrite in the samples were determined by interpolating absorbance from the standard curve and multiplying by the dilution factor (1:2).

**Immunohistochemistry.** Immunohistochemical detection of NT adducts in the mesentery was performed on formalin-fixed paraffin-embedded tissue sections (5 μm). Staining was performed by using a combination of offline manual procedures and a Ventana Discovery XT automated slide stainer.
The values presented are the average of the individually scored parameters within 0.05. Statistical analysis was not conducted on the histopathology data. The doses, across experiments. Differences were considered significant when matched pairs analysis allowed for detection of differences, at similar CI-1044 comparisons with a post hoc minimal mixed perivascular leukocyte infiltration (Fig. 1A) and utilized in toxicology studies. These findings consisted of considered to be background events not uncommonly found in rodents utilized in toxicology studies. These findings consisted of minimal mixed perivascular leukocyte infiltration (Fig. 1A) and small foci of mixed to mononuclear inflammation separating and surrounding mesenteric adipocytes (Fig. 1B). Treatment with CI-1044 alone at doses of 10, 20, and 40 mg/kg/day for 3 days resulted in a dose-related increase in the incidence and severity of perivasular and mesenteric inflammation as well as vascular injury. Vascular injury was characterized by one or more of the following changes including endothelial hypertrophy, vacuolation or necrosis, and/or medial hemorrhage, fibrinoid necrosis, or vasculitis. At the lowest dose of CI-1044, microscopic findings consisted primarily of mixed inflammation, hemorrhage, and leukocytic cavementing. The incidence of each finding was variable between each animal within the group. The severity ranged from minimal to mild and was most prevalent around postcapillary venules and within mesenteric adipose tissue. Mesenteric arteries were less frequently and less severely affected. Similar microscopic findings were present in the mid-dose (20 mg/kg/day) group, although the incidence of each finding was increased, affecting all or most animals within the group, and the severity was increased ranging from minimal to moderate. In addition, mild to moderate edema and fibroblast

**Statistical Analysis.** Data were reported as the mean ± SE. The Bartlett’s test was used to test for homogeneity of variance and to select the type of analysis to be conducted. SN data were analyzed using a Kruskal-Wallis one-way ANOVA (Kruskal and Wallis, 1952) followed by intergroup pairwise comparisons with a post hoc test described by Wilcoxon and Wilcox (1964). A matched pairs analysis allowed for detection of differences, at similar CI-1044 doses, across experiments. Differences were considered significant when p < 0.05. Statistical analysis was not conducted on the histopathology data. The values presented are the average of the individually scored parameters within four anatomical substructures (measured on an ordinal scale from 0 to 4).

**RESULTS**

**Histopathology**

Microscopic findings were assessed in mesenteries harvested 24 h following the last dose for each treatment group. Microscopic findings in vehicle-treated animals were considered to be background events not uncommonly found in rodents utilized in toxicology studies. These findings consisted of minimal mixed perivascular leukocyte infiltration (Fig. 1A) and small foci of mixed to mononuclear inflammation separating and surrounding mesenteric adipocytes (Fig. 1B). Treatment with CI-1044 alone at doses of 10, 20, and 40 mg/kg/day for 3 days resulted in a dose-related increase in the incidence and severity of perivasular and mesenteric inflammation as well as vascular injury. Vascular injury was characterized by one or more of the following changes including endothelial hypertrophy, vacuolation or necrosis, and/or medial hemorrhage, fibrinoid necrosis, or vasculitis. At the lowest dose of CI-1044, microscopic findings consisted primarily of mixed inflammation, hemorrhage, and leukocytic cavementing. The incidence of each finding was variable between each animal within the group. The severity ranged from minimal to mild and was most prevalent around postcapillary venules and within mesenteric adipose tissue. Mesenteric arteries were less frequently and less severely affected. Similar microscopic findings were present in the mid-dose (20 mg/kg/day) group, although the incidence of each finding was increased, affecting all or most animals within the group, and the severity was increased ranging from minimal to moderate. In addition, mild to moderate edema and fibroblast

**FIG. 1.** Photomicrographs showing alterations of vascular injury in rats treated for 3 days with vehicle, CI-1044 alone, or CI-1044 in conjunction with a modulator of NO. Staining with hematoxylin and eosin in all slides. (A) Vehicle control. Mesenteric muscular artery. Minimal, focal, and periarterial neutrophilic infiltrate (arrows). Scale bar = 20 μm. (B) Vehicle control. Mesentry. Minimal, focal, neutrophilic, and histiocytic inflammatory infiltrate (arrows). Scale bar = 20 μm. (C) CI-1044, high dose. Mesentery. Marked, multifocal, necrotizing, and hemorrhagic arteritis accompanied by moderate, locally extensive, acute periarterial inflammation, and hemorrhage. Scale bar = 500 μm. (D) CI-1044, high dose. Mesenteric muscular artery. Marked, focal, necrotizing, and hemorrhagic arteritis accompanied by marked, diffuse, acute periarterial and mesenteric inflammation, and hemorrhage (arrows). Scale bar = 500 μm. (E) CI-1044 + SIN-1, high dose. Mesentery. Marked, diffuse, necrotizing, and hemorrhagic arteritis accompanied by marked, diffuse, acute periarterial and mesenteric inflammation, and hemorrhage. Scale bar = 500 μm. (F) CI-1044 + SIN-1, high dose. Mesentry. Marked, diffuse, necrotizing, and hemorrhagic arteritis accompanied by marked, diffuse, acute periarterial and mesenteric inflammation, and hemorrhage (arrows). Scale bar = 50 μm. (G) CI-1044 + L-NAME, high dose. Mesentry. Minimal, multifocal, acute, periarterial, perivenous and mesenteric inflammation, and hemorrhage. Scale bar = 50 μm.
Lesion Scores for Mesenteric Anatomical Substructures

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Note: Values are average ± SE of the mean for the sum of the lesion scores in each tissue group. CI-10, CI-1044 10 mg/kg/day; CI-20, CI-1044 20 mg/kg/day; CI-40, CI-1044 40 mg/kg/day; LN, L-NAME 60 mg/kg/day; MAI, mesenteric artery intima; MAA, mesenteric artery adventitia; MAM, mesenteric artery media; MV, mesenteric vein; NOM, nitric oxide modulator; PDEi, phosphodiesterase inhibitor; SIN-1, 30 mg/kg/day; VH, vehicle.

Microscopic findings in animals treated with the NO donor SIN-1 alone were similar to those treated with vehicle alone and consisted of minimal mixed perivascular leukocyte infiltration and leukocyte pavementing. In contrast, coadministration of SIN-1 with CI-1044 considerably enhanced mesenteric inflammation and vascular injury compared with animals receiving CI-1044 alone. Moreover, venules and arterioles were equally affected and the incidence was increased. A dose of 10 mg/kg/day CI-1044 in combination with SIN-1 produced marked arterial medial fibrinoid necrosis and mild to moderate vasculitis. In addition, mesenteric and perivascular inflammation, edema, hemorrhage, and fibroblast proliferation ranged from mild to marked. Inflammation and/or hemorrhage/edema affected all animals in the group, whereas other changes were less frequent affecting 40–80% of the animals in this group. Treatment with 20 mg/kg/day CI-1044 in conjunction with SIN-1 resulted in a greater incidence and severity of these findings. Notably, all animals in the group were affected by numerous microscopic findings involving venules, arteries, and the mesentery as a whole. The arteries were characterized by mild to marked arterial medial fibrinoid necrosis, minimal to moderate endothelial hypertrophy, necrosis and leukocyte pavementing, and marked perivascular hemorrhage/edema, inflammation, and fibroblast proliferation. Venules were characterized by mild to marked leukocyte pavementing, endothelial hypertrophy and necrosis, perivascular edema, hemorrhage, inflammation, and fibroblast proliferation. The mesentery was characterized by marked inflammation, hemorrhage/necrosis, and fibroblast proliferation. At the highest CI-1044 dose tested in conjunction with SIN-1, microscopic findings were similar to the mid-dose group, but the incidence and severity of fibrinoid necrosis was greater (Figs. 1E and 1F).

Treatment with CI-1044 in conjunction with the NOS inhibitor L-NAME had the overall effect of mitigating microscopic findings associated with exposure to CI-1044 alone (Fig. 1D). For example, there was no indication of medial necrosis in any of the groups receiving CI-1044 plus L-NAME. In contrast, 1/5
low-dose, 3/5 mid-dose, and 4/5 high-dose animals receiving CI-1044 alone exhibited medial necrosis/hemorrhage. The inflammatory response, although still present, was also reduced when treated with 40 mg/kg/day CI-1044 plus L-NAME as compared with treatment with 40 mg/kg/day CI-1044 alone. Coadministration of CI-1044 at 40 mg/kg/day with L-NAME also produced considerable decreases in the incidence and severity of venous medial necrosis and mesenteric injury (reactive fibroblasts) as compared with that which was observed to occur in response to treatment with 40 mg/kg/day CI-1044 alone.

The selective iNOS inhibitor MEG had no effect on the severity of the CI-1044–induced injury.

**Serum Nitrite**

The treatment-related changes in the levels of SN are presented in Figure 2 in relation to the changes in histopathology scores. Exposure to CI-1044 alone produced robust dose-related elevations in SN compared with the vehicle control group. A significant increase was observed at the 40 mg/kg/day dose level, where the percent increase was 312% compared with the controls. When the NO donor molecule SIN-1 was coadministered with CI-1044, a similar trend of dose-related increases in SN was observed. As expected, the SN levels were greater at each CI-1044 dose level in the CI-1044 plus SIN-1 study compared with the CI-1044–alone study. Compared with the SIN-1–only group (the appropriate vehicle control group for the CI-1044 plus SIN-1 study), the significant increase that was observed again occurred at the 40 mg/kg/day CI-1044 dose level; the percent increase was 630% over the SN value for SIN-1 alone. Coadministration with the NOS inhibitor, L-NAME prevented CI-1044 (at the two lowest groups tested) from inducing an increase in SN values. In fact, SN levels for animals receiving CI-1044 at 10 or 20 mg/kg/day were decreased by 3 and 44%, respectively, when compared with animals receiving L-NAME alone (the appropriate vehicle control group for the CI-1044 plus L-NAME study). However, L-NAME did not prevent a modest rise in SN at the highest CI-1044 dose tested. Although the increase was not significant, there was a twofold increase in SN in the group receiving 40 mg/kg/day CI-1044 plus L-NAME compared with the group receiving L-NAME alone.

For CI-1044 alone, there is a positive relationship between SN and histopathology score. For 10 mg/kg/day of CI-1044, the histopathology score is increased, although the SN value is not different from CI-1044 alone. At doses of 20 or 40 mg/kg/day of CI-1044, SIN-1 increased both total SN and histopathology score as compared with CI-1044 alone.

The iNOS inhibitor MEG had no effect on the CI-1044–induced elevation in SN values (data not shown).

**Immunohistochemistry**

Changes in immunoreactivity to anti-nitrotyrosine are shown in Figure 3. There was virtually no nitrotyrosine detected in the mesenteries from rats treated with the vehicle control (Fig. 3A). Treatment with the PDE4 inhibitor CI-1044 at a dose of 40 mg/kg/day for 3 days resulted in positive immunoreactivity for nitrotyrosine in the vascular ECs and the perivascular inflammatory cells that were present (Fig. 3B). Coadministration of SIN-1 with CI-1044 resulted in strong positive immunoreactivity in mesenteric vascular ECs and inflammatory cells (Fig. 3C).

![FIG. 2. Effects of L-NAME or SIN-1 on the relationship between total serum nitrite and histopathological findings after three daily doses of orally administered CI-1044 (10, 20, or 40 mg/kg/day). Results are expressed as mean ± SE. *p < 0.05 significantly different from the respective within-study control group, † p < 0.05 significantly different from the same CI-1044 + L-NAME–dose group.](image-url)
Coadministration of L-NAME with CI-1044 resulted in essentially no immunoreactivity to mAb nitrotyrosine (Fig. 3D).

**DISCUSSION**

The present study demonstrated that the extent of PDE4 inhibitor CI-1044–induced mesenteric vascular injury in the SD rat was modulated by altering in vivo NO levels. CI-1044–induced vascular toxicity consisted of medial hemorrhage and fibrinoid necrosis of arterioles and arteries and perivascular inflammation. In addition, marked leukocyte pavementing was observed in the arterial intima along with mixed inflammatory infiltrates in the adventitia. Profound edema was also observed in the adventitia as well as in the mesenteric connective tissue. Similar morphology of PDE4 inhibitor–induced mesenteric injury was observed in previous studies (Dagues et al., 2007; Zhang et al., 2008). The dose-related increases in histopathology following treatment with the PDE4 inhibitor CI-1044 also resulted in increasing levels of SN and stronger immunostaining for NT. Mesenteric arterial injury and inflammation was markedly increased in frequency and severity in the animals treated with CI-1044 and SIN-1 when compared with the animals treated with SIN-1 or CI-1044 alone. Mesenteric vascular injury and inflammation in animals treated with a combination of CI-1044 and L-NAME was reduced in frequency and severity compared with animals receiving CI-1044. These results suggest that the amount of nitric oxide is directly linked to the mechanism of injury in DIVI. The ability of L-NAME to inhibit DIVI suggests that the activity of an NOS is involved. The roles of both eNOS and iNOS in the physiology of blood vessels are well established (Raghavan and Dikshit, 2004). The absence of effect of the iNOS inhibitor MEG is consistent with the hypothesis that the effect of CI-1044 could be mediated through the constitutively expressed eNOS protein.

Once formed, NO rapidly diffuses through tissue and is readily scavenged by blood in the vasculature (Butler et al., 1998) where it reacts with oxyhemoglobin and is converted to nitrate. Taken together with NO’s propensity to react with superoxide, the half-life of NO in vivo is < 1 s (Hakim et al., 1996), which makes detection of the native molecule in situ prohibitively difficult. SN has been shown to sensitively reflect endothelial NO formation (Kelm et al., 1999). SN has also been shown to increase in response to treatment with an SCH 351591 (Weaver et al., 2008, 2010), a PDE4 inhibitor that is structurally unrelated to CI-1044.

At physiological levels, nitric oxide is a potent bioregulatory agent that is intimately involved in maintaining homeostasis of the cardiovascular system (Papapetropoulos et al., 1999). However, there is evidence from a variety of sources that suggest that elevated levels of NO can be both protective and deleterious. The role of NO in ischemia reperfusion (IR) injury, for example, is somewhat controversial in that there are reports that NO can reduce the damage and cytotoxicity from reactive oxygen species that form during IR (Wink et al., 1993). On the
other hand, NO reacts rapidly with a key mediator of IR injury—superoxide to form the highly reactive and damaging peroxynitrite (Crow and Beckman, 1995), which has been linked to numerous diseases (Pacher et al., 2007). Nitric oxide is also injurious due to its inherent ability to deaminate nucleotides and nucleic acids (Wink et al., 1991). Peroxynitrite formation in vivo leaves behind the distinct footprint of nitrotyrosine adducts (Crow and Beckman, 1995). Utilizing immunohistochemical methods to measure nitrotyrosine, nitratetive stress has been shown to occur in PDE4-induced mesenteric vascular injury in rats (Slim et al., 2003; Zhang et al., 2008) in a similar pattern to that observed in this study.

Cell death via apoptosis and/or necrosis appears to be a common downstream event in PDE4 inhibitor-induced vascular injury. For example, apoptosis following PDE4 inhibitors treatment has been visualized via TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining (Slim et al., 2003; Zhang et al., 2008). This measure of cell death explains the observation that DIVI is sometimes associated with a significant loss of vascular ECs. Furthermore, cell death may also explain the observation of fibrin insudation in the mesenteric arterial walls and medial hemorrhage. We observed the morphological hallmarks of apoptosis, chromatin condensation, and margination areas where we detected the loss of vascular endothelial and smooth muscle cells. Elevation of NO has also been associated with cell death by apoptosis or by necrosis mediated through poly ADP ribose polymerase activation. Depending on the system and conditions examined, a given cell population may use one, the other, or both pathways (Pacher et al., 2007). Even within apoptotic systems, the effect of NO can protect from apoptosis or promote apoptosis, depending on the concentration (Dimmeler and Zeiher, 1997; Thomas et al., 2008).

Nitric oxide is produced by one of the three nitric oxide synthases (NOS). The endothelial isoform (eNOS or NOS3) is constitutively expressed in vascular ECs (Sessa, 2004). The PDE4 protein is responsible for hydrolysis of cAMP to control signaling through a variety of pathways. Inhibition of this protein in rat aortic rings in vitro has been shown to result in a significant rise in intracellular cAMP but not cGMP (Eckly and Lugnier, 1994). Elevation of cAMP has been shown to result in activation of eNOS with production of NO in several systems. Treatment of canine cardiac microvessels with the adenylyl cyclase activator forskolin or with 8-Br-cAMP resulted in increased release of NO. This was blocked by the NOS inhibitor L-NAME and by the PKA inhibitor Rp-cAMP (Zhang and Hintze, 2006). In bovine aortic ECs, treatment with the adenylyl cyclase activator genistein results in a dose-dependent increase in NO and in phosphorylation of eNOS at Ser-1179. This effect was blocked by the PKA inhibitor H-89 but not by inhibitors of PI3K or of ERK/MAPK. Furthermore, treatment with genistein resulted in a rise in intracellular cAMP levels (Liu et al., 2004). Treatment of human aortic ECs with the PDE4i rolipram, the PDE3i cilostazol, or with forskolin resulted in a dose-dependent increase in NO release. This release was blocked by L-NAME and by the PKA inhibitor, PKAI, which also blocked the cilostazol-induced eNOS phosphorylation at Ser-1177 (Hashimoto et al., 2006). Both eNOS and PKA have been observed to be colocalized in rat aortic endothelial luminal lamellipodia (Heijnen et al., 2004). Taken together, these data are consistent with the hypothesis that the PDE4i Cl-1044 induces production of NO through PKA-mediated activation of eNOS.

In addition to PDE4-selective agents, other PDE isomser-selective inhibitors (e.g., PDE3 inhibitors) as well as non-selective PDEi also cause similar vascular injury (Collins et al., 1988; Zhang et al., 2006). The PDE3i also inhibit the degradation of cAMP, and it is therefore plausible to have a mechanism of action similar to that of PDE4i (Polson and Strada, 1996).

There are also numerous other drugs and drug classes with great therapeutic promise that have been shown to cause DIVI in laboratory animals. These include dopamine (DA1) agonists (Dalmas et al., 2008), endothelin receptor antagonists (Albassam et al., 2001), K+-channel blockers (van Vleet et al., 1984), and adenosine receptor agonists (Enerson et al., 2006). The histopathology we observed to occur in response to Cl-1044 treatment is typical of the numerous classes of drugs that cause DIVI. The presumption is that there may be several mechanistic pathways that could contribute to DIVI in in vivo injury that may be occurring in rats. Previous research has demonstrated that elevation of serum nitrate was one of many changes associated with DIVI. The results in this study suggest that the nitric oxide pathway is more directly involved in the pathogenesis of DIVI. If confirmed and extended into other non-PDE drug classes that also cause DIVI, it might suggest that elevated serum nitrate could potentially be used as a mechanistically linked biomarker of this type of injury.

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