Effects of Peroxisome Proliferator–Activated Receptor Ligands in Modulating Tissue Factor and Tissue Factor Pathway Inhibitor in Acutely Symptomatic Carotid Atheromas

Jonathan Golledge, MChir; Simone Mangan, BS; Paula Clancy, PhD

Background and Purpose—Severely stenotic, symptomatic carotid atheromas are associated with a high risk of stroke in the short term. Although carotid endarterectomy is effective in reducing this stroke risk, it is frequently not applied within the time window for significant benefit. We investigated the effect of peroxisome proliferator–activated receptor (PPAR)-α and -γ ligands in acutely modifying tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in unstable carotid atheromas.

Methods—During a 3-year period, 64 patients who had experienced a transient ischemic attack or stroke with good recovery within 6 weeks before surgery and 12 asymptomatic patients with a >70% carotid stenosis were recruited. The expression of PPAR-α and -γ was investigated in endarterectomy samples. The effects of the PPAR-α and -γ ligands fenofibrate and rosiglitazone were investigated in cell culture experiments. Targeted biopsy specimens from endarterectomy samples (n=48) were incubated with medication for 4 days. TF and TFPI were assessed by immunohistochemistry, Western blot analysis, flow cytometry, and activity assays.

Results—PPAR-γ1 but not -α was downregulated in atheromas removed from patients with recent symptoms and no evidence of diabetes. Fenofibrate but not rosiglitazone impaired the induction of TF in human endothelial cells and reduced resting levels of TF activity in vascular smooth muscle cells. Rosiglitazone but not fenofibrate increased TFPI secretion from human endothelial cells. Both fenofibrate (100±18.7% to 56.6±8.8%, P=0.005; 0.2664±0.0696 to 0.1771±0.0310, P=0.02) and rosiglitazone (100±22% to 88.3±20%, P=0.02; 0.3113±0.0729 to 0.2287±0.0415, P=0.04) reduced TF expression and activity, respectively, in atheroma biopsy specimens. A low expression of TFPI was found in atheroma biopsy specimens with little evidence of TFPI activity.

Conclusions—This study suggests that both PPAR-α and -γ ligands have beneficial effects in acutely reducing TF in unstable carotid atheromas. (Stroke. 2007;38:1501-1507.)

Key Words: atherosclerosis, symptomatic ▪ carotid artery ▪ tissue factor
rupture, exposure of TF leads to formation of the TF–factor VII (FVII)/FVIIa complex that drives generation of FXa and ultimately, thrombosis. The primary inhibitor of TF is tissue factor pathway inhibitor (TFPI), which complexes with FXa-TF/FVIIa to prevent further generation of FXa. The balance between TF and TFPI activity within atheroma is an important determinant of thrombus formation after plaque rupture and therefore, the clinical sequelae. In this study, we investigated the potential of medication that ligates the nuclear transcription factor peroxisome proliferator–activated receptors (PPARs) -α and -γ in acutely modulating TF and TFPI within human carotid atheromas.

Methods

Study Design
The study was carried out in 3 phases. First, we studied the expression of TF, TFPI, and PPAR-α and -γ in carotid atheromas from 16 symptomatic and 12 asymptomatic patients (the Table). Second, we assessed the effect of the PPAR-α and -γ ligands fenofibrate and rosiglitazone, respectively, on TF and TFPI expression and activity in cell culture. Finally, we examined the influence of a number of different PPAR-α and -γ ligands (fenofibrate, gemfibrozil, rosiglitazone, and pioglitazone) on the expression and activity of TF and TFPI in human carotid atheroma explants from patients with a recent TIA or stroke.

Patients
This study was approved by the local ethics committees, and informed consent was obtained from participating patients. Entry criteria were as follows: (1) a TIA or stroke ≤6 weeks before carotid endarterectomy (phases 1 and 3); (2) no previous neurologic symptoms before carotid endarterectomy (phase 1 only); and (3) internal or common carotid artery (CCA) stenosis >70% defined by velocity criteria on duplex scanning, as previously described. Exclusion criteria included patients with amaurosis fugax alone or nonfocal, atypical, or distant neurologic symptoms and patients not receiving a statin or antiplatelet medication. Clinical, imaging, and serum characteristics of the patients were prospectively measured and recorded, as previously described.

Specimens
A conventional endarterectomy was performed, and specimens were immediately placed in cold culture medium and brought to the laboratory for experimental work. With sterile techniques, specimens were cut from the proximal internal carotid artery (PICA) and CCA endarterectomy sites for primary assessment by immunohistochemistry (4 patients, 4 biopsy specimens), Western blotting (24 patients, 24 biopsy specimens), or explant culture (48 patients, 144 biopsy specimens). Each specimen was used for immunohistochemistry, Western blotting (phase 1), or explant culture (phase 3 alone) to ensure that the diseased site from the PICA and CCA within the specimen was obtained for the assessment in question.

Explant Culture
Incubation of targeted endarterectomy biopsy specimens with PPAR-α or -γ ligands was carried out in vitro as previously described. In preliminary studies, we had established that (1) the carotid endarterectomy samples remained viable in culture for at least 5 days, as assessed by histology, immunohistochemistry, cytokine release, and measurement of tissue ATP concentration; (2) viability was not influenced by incubation with therapeutic concentrations of PPAR ligands (explants

### Table: Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Initial Study</th>
<th>Medication Study</th>
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IHD indicates ischemic heart disease; PSV, peak systolic velocity on duplex imaging; WBC, white blood count; and CRP, C-reactive protein.
continued to secrete cytokines and maintained ATP concentrations); and (3) the concentration and activity of TF were similar in adjacent biopsy specimens removed from the PICA or CCA of an endarterectomy sample, ie, 1a (or 2a) compared with 1b (or 2b) (Figure 1). The coefficient of variation of TF activity in 6 paired biopsy specimens was <5%. Paired biopsy specimens from the macroscopically diseased portion of the PICA (1a and 1b) and CCA (2a and 2b) were placed in tissue-culture wells, intima up, in 1 mL of medium and incubated at 37°C in a humidified 5% CO2 atmosphere (Figure 1). Biopsy specimens were stabilized in culture for 24 hours before replacement of the culture medium with (experimental sample 1b or 2b) or without (control 1a or 2a) therapeutic concentrations of PPAR-α or -γ ligand. Thus, 1 sample from each site (1b and 2b) was incubated in the presence of a PPAR-α (fenofibrate 10 μmol/L or gemfibrozil 50 μmol/L) or a PPAR-γ (rosiglitazone 10 μmol/L or pioglitazone 5 μmol/L) ligand, whereas the other (1a and 2a) acted as a control. The dose of drug was based on published safe serum levels of these medications achieved in patients on treatment.14–17 For example, the serum concentration of pioglitazone in patients receiving repeated doses of this medication has been recorded as 5 μmol/L.16 The culture medium was replaced every 48 hours, and the harvested medium was centrifuged (30 000g for 30 minutes at 4°C) to remove particulate debris and stored frozen in aliquots at −80°C until analyzed for matrix metalloproteinase activity.18 After 5 days in culture, explants were stored frozen at −80°C for later assessment of TF and TFPI expression or activity.

Cell Culture
Human aortic endothelial cells (HAECs) and vascular smooth muscle cells (VSMCs) were purchased from Cambrex Bio Science Pty Ltd. Cells were grown to confluence and then passaged into 24-well plates (104 cells/well) for experiments. TF is expressed in resting VSMCs but not HAECs. To assess the effect of PPAR medication on TF expression in the endothelium, cells were incubated in the drug of interest for 2 hours before stimulation with tumor necrosis factor-α (100 U/mL). In a preliminary time-course study, maximum stimulation of TF protein was demonstrated to be 6 hours after induction. Thus, for medication studies, cells were harvested 6 hours after induction. Because TFPI is expressed by unstimulated cells, no induction was required for assessment of this protein. TFPI studies were planned for a 4-day incubation period to match the explant studies.

Immunohistochemistry
Selected serial cryostat sections from biopsy specimens of the PICA of symptomatic patients were stained by the Envision immunoperoxidase method with antibody titers optimized in preliminary studies to CD31 (Dako, JC70A, 4.5 μg/mL), α-actin (Dako, IA4, 0.7 μg/mL), CD68 (Dako, KP1, 1.25 μg/mL), PPAR-α (Santa Cruz, N19, 4 μg/mL), PPAR-γ (Santa Cruz, H100, 4 μg/mL), TF (American Diagnostica, TFP10H10, 5 μg/mL), and TFPI (American Diagnostica, 4903, 5 μg/mL).12,18

Western Blotting
Western blotting was used to compare PPAR-γ and -α expression in atheromas removed from recently symptomatic and asymptomatic patients; PPAR-γ expression in atheromas removed from patients with and without diabetes; and TF and TFPI expression in control and treated explants, as previously described.12–18 In preliminary studies, antibody titers (PPAR-α and -γ, Santa Cruz, N19 and H100, 0.4 μg/mL; TF, American Diagnostica, TFP10H10, 0.5 μg/mL; TFPI, American Diagnostica, 4903, 1 μg/mL) and blotting conditions were optimized. Thirty micrograms of protein (measured by the Bradford technique; Bio-Rad) from samples to be compared (ie, symptomatic and asymptomatic, diabetic and nondiabetic, or treated and control explants) were always analyzed on the same Western blot to ensure comparable exposure conditions. Densitometry was used to quantify the protein of interest, and results are presented as mean±SE for both relative density units and ratios for the samples of interest (symptomatic, diabetic, or treated) and controls (asymptomatic, nondiabetic, or untreated).12

TF and TFPI Activity
TF activity was assessed in cell culture with a commercial chromogenic assay that measures the generation of FXa (American Diagnostica).19 TFPI activity was assessed with a commercial assay that measures the ability of TFPI present in the test sample to inhibit a constant and known amount of TF/FVIIIa (American Diagnostica).20 TF and TFPI activities in atheroma samples were measured as previously described.21 The intra-assay and interassay coefficients of variation for these assays were between 3% and 6%.

Flow Cytometry
After culture with medication or control, HAECs were collected in wash buffer (0.1% phosphate-buffered saline/horse serum albumin and 0.1% NaN3), incubated on ice with a monoclonal antibody to TF (American Diagnostica, TF910H10, 3 μg/mL) or IgG negative control (Dako, DAKG01, 3 μg/mL) for 30 minutes, washed, and then incubated for another 30 minutes with a secondary fluorescein isothiocyanate–labeled anti-mouse IgG (Dako, F0479, 20 μg/mL) before fluorescence-activated cell sorting.18

Zymography
The protease activity of conditioned media removed from control and experimental explants at 24, 72, and 120 hours was assessed by gelatin zymography, as previously described.18

Data Analysis
For comparison of PPAR-γ and -α expression in specimens removed from symptomatic and asymptomatic patients or diabetics and nondiabetics, results are presented as mean±SE for both density units and relative ratios; these were analyzed by the Mann–Whitney U test.12,18 For assessment of the effect of medication on TF surface expression by HAECs, data were compared by the Kruskal–Wallis test. For assessment of the effect of medication on TF activity in
VSMCs, results were compared with the Mann Whitney U test. TF and TFPI expression in explants is presented as mean±SE for both density units and relative ratios of paired experimental and control samples; these were compared with the Wilcoxon signed-rank test.

Results

Patients
Between July 2003 and 2006, 76 patients with a recent history of TIA (n=46) or stroke (n=18) or who were asymptomatic (n=12) and with a >70% carotid stenosis who underwent carotid endarterectomy were recruited (the Table).

PPAR-α and -γ Are Present in Carotid Atheromas
In an initial study of carotid atheromas removed from 28 patients (16 symptomatic and 12 asymptomatic; the Table), PPAR-α and -γ were demonstrated within biopsy specimens by immunohistochemistry and Western blotting (Figure 2A). The expression of PPAR-γ1 but not the -γ2 isoform was noted to be greater in symptomatic patients who had diabetes compared with those who did not (mean density units 56 067±3062 compared with 33 709±5628, P=0.04, for -γ1; 19 833±2206 compared with 21 212±1508, P=0.70, for -γ2). This difference was not evident when PPAR-γ1 and -γ2 isoforms were compared in diabetic patients without symptoms (eg, for PPAR-γ1, 14 105±780 compared with 14 799±2800, P=0.91). In view of this difference between patients with and without diabetes, we separately compared the expression of PPAR-γ and -α in diabetics and nondiabetics with and without symptoms (Figure 2). In patients without diabetes, the expression of PPAR-γ1 was 2-fold less in atheromas from patients with recent symptoms of cerebral embolization (Figure 2B). No association of symptoms with PPAR-γ or -α expression was noted in the atheromas from patients with diabetes (Figure 2C).

Figure 2. Expression of PPAR-α and -γ in carotid atheromas in relation to diabetes and symptoms. A, Representative Western blot showing PPAR-γ1 (upper band) and PPAR-γ2 (lower band) expression in carotid atheromas removed from patients without diabetes either with recent focal neurologic symptoms (left panel) or asymptomatic (right panel). B and C, Relative expression of PPAR-γ1, -γ2, and -α in carotid atheromas in relation to diabetes (G, patients with, and B, those without, diabetes) and symptoms. Mean density units for diabetics were as follows: PPAR-γ1 42 229±10 871 and 41 112±5945, P=0.63; PPAR-γ2 15 243±1427 and 15 366±2647, P=0.42; PPAR-α 16 274±5448 and 18 688±5288, P=0.87 for symptomatic (Ⅻ) and asymptomatic (□) patients, respectively (n=6 for each measurement). For nondiabetics, corresponding values were as follows: PPAR-γ1 14 083±3732 and 34 711±4947, P=0.009; PPAR-γ2 12 989±3702 and 17 997±5326, P=0.59; PPAR-α 36 319±6138 and 36 543±4065, P=0.82 for symptomatic and asymptomatic patients, respectively (n=6 for each measurement).
Effect of PPAR Ligands in Cell Culture

Using immunohistochemistry, we identified marked staining for TF in the lipid core and activated endothelium in atheromas removed from patients with recent symptoms (data not shown). Initially, we assessed the ability of PPAR ligands to modulate induction of the surface expression of TF in HAECs by fluorescence-activated cell sorting. We found that the PPAR-α ligand fenofibrate (n=3, P=0.04) but not the PPAR-γ ligand rosiglitazone (n=3, P=0.9) dose-dependently inhibited upregulation of TF by the cytokine, tumor necrosis factor-α. For example, the surface expression of TF was reduced to 76.3±2.8% of control in HAECs exposed to tumor necrosis factor-α in the presence of 30 μmol/L fenofibrate.

We demonstrated significant basal levels of TF in human VSMCs and investigated the effects of incubating them with PPAR ligands for 4 days. Fenofibrate (10 μmol/L; activity equivalent to 12.58 pmol/L of TF compared with 17.54 pmol/L for control, P=0.01) but not rosiglitazone (10 μmol/L; activity equivalent to 15.17 pmol/L of TF compared with 17.54 pmol/L for control, P=0.5) significantly inhibited the TF activity of VSMCs. We demonstrated TFPI activity to be present primarily in secretions from HAECs, with no activity in VSMCs. Rosiglitazone (10 μmol/L) but not fenofibrate (10 μmol/L) stimulated TFPI secretion by HAECs. The mean TFPI activity of conditioned media generated for 48 hours increased from 96.2±7.1 (day 2) and 107.4±7.2 (day 4) to 126.6±3.7 (day 2, P=0.006) and 128.9±2.7 (day 4, P=0.01) mU/mL in the presence of rosiglitazone (n=6), whereas secretion in the presence of fenofibrate (116.8±5.9, P=0.1), at day 2; 118.8±6.8, P=0.2, at day 4; n=6) was not significantly altered.

Effect of PPAR Ligands on TF and TFPI Expression in Carotid Atheromas In Vitro

To investigate the effect of PPAR ligands on TF and TFPI within human atheromas, we initially used endarterectomy samples from 24 patients with recent symptoms and incubated paired samples from the PICA and CCA with medication or control (a total of 12 treated and control biopsy specimens for each medication). At the end of the incubation period, the expression of TF and TFPI was assessed in comparison between treatment and control pairs by Western blot analysis. Incubation of atheroma biopsy specimens in the presence of PPAR-α ligands fenofibrate (10 μmol/L) and gemfibrozil (50 μmol/L) for 4 days led to a significant reduction in the expression of TF (Figure 3). Rosiglitazone but not pioglitazone also significantly reduced TF expression. By immunohistochemistry and Western blotting, a low expression of TFPI was demonstrated in carotid atheromas. Surprisingly, incubation of atheromas with rosiglitazone resulted in a reduction of TFPI expression (Figure 4). Other medications had no effect on TFPI expression.

Figure 3. Relative expression of TF in control (◊) and treated (□) carotid atheromas assessed by Western blot analysis. Shown are the mean±SE of densitometry results for treated relative to control samples. Rosiglitazone (mean density units 6332±790 compared with control 3950±623, P=0.02) but not fenofibrate (mean density units 5208±651 compared with control 4507±539, P=0.12), gemfibrozil (mean density units 4140±275 compared with control 3811±333, P=0.15), and pioglitazone (mean density units 4834±788 compared with control 3457±472, P=0.17) reduced TFPI expression in carotid atheromas.

Figure 4. Relative expression of TFPI in control (◊) and treated (□) carotid atheromas assessed by Western blot analysis. Shown are the mean±SE of densitometry results for treated relative to control samples. Rosiglitazone (mean density units 6332±790 compared with control 3950±623, P=0.02) but not fenofibrate (mean density units 5208±651 compared with control 4507±539, P=0.12), gemfibrozil (mean density units 4140±275 compared with control 3811±333, P=0.15), and pioglitazone (mean density units 4834±788 compared with control 3457±472, P=0.17) reduced TFPI expression in carotid atheromas.
PPAR-α and -γ Ligands Have Similar Effects on TF Activity of Carotid Atheromas In Vitro

Given the marked effects of fenofibrate and rosiglitazone on the expression of TF and TFPI in carotid atheromas, we next studied the effects of these medication on TF and TFPI activity in additional atheroma biopsy specimens sampled from the PICA of 24 recently symptomatic patients (the Table). Both fenofibrate and rosiglitazone reduced TF activity of carotid atheromas. Fenofibrate reduced the rate of FXa generation from 0.2664±0.0696 to 0.1771±0.0310 U · min⁻¹ · mg⁻¹ protein (P=0.02). Rosiglitazone reduced the rate of FXa generation from 0.3113±0.0729 to 0.2287±0.0415 U · min⁻¹ · mg⁻¹ protein (P=0.04). We demonstrated no drop in TF activity in the presence of a TFPI blocking antibody. For example, the mean reduction in TF activity in 24 samples assessed in the fenofibrate experiment was 0.0009±0.0001 U · min⁻¹ · mg⁻¹ protein, which represents only a 3% reduction in TF activity. No reduction was noted in 24 samples examined in the rosiglitazone experiment. The changes induced by PPAR ligands in TF and TFPI were not accompanied by changes in matrix metalloproteinase secretion from explants (supplemental Figure I, available online at http://stroke.ahajournals.org).

Discussion

After a clinical event, an atherosclerotic plaque is much more likely to be associated with a second event. Research has been directed at medications to stabilize the fibrous cap and thereby reduce the risk of plaque rupture, eg, by downregulating proteinases and increasing collagen content within the fibrous cap. In this study, we concentrated on another potentially important aspect of plaque stability, ie, the likelihood of the plaque to stimulate thrombus formation when rupture occurs. The most significant finding of this study is the ability of both fenofibrate and rosiglitazone to acutely reduce the TF activity of atheromas by downregulating TF (Figure 3). Fenofibrate treatment for 4 days convincingly reduced the mean expression of TF in atheromas by 43% and was associated with a decrease in TF activity of 33%. Rosiglitazone treatment for the same duration led to a smaller 12% and 26% mean reduction in TF expression and activity, respectively.

Previous investigators have assessed the influence of PPAR-γ and -α on TF activity in monocytes or endothelial cells. Interestingly, in this study, we demonstrated that fenofibrate inhibited the induction of TF in HAECs and reduced the basal TF activity in VSMCs, suggesting the cellular targets for the effects that we demonstrated in carotid atheromas. However, we were unable to demonstrate any effects of the PPAR-γ ligand rosiglitazone on HAECs or VSMCs. The benefits of rosiglitazone demonstrated in carotid atheromas might result from the effects on macrophage-derived or noncellularly associated TF. Alternatively, the effects of medication in atheromas may be more complex than we are able to model in cell culture. The collection of different cytokines within the atheroma may alter cellular responses. In addition, clinical factors may modulate the effects of medications. Evidence in support of the latter hypothesis was provided by our assessment of PPAR concentrations in carotid atheromas. We demonstrated that the concentration of the transcription factor PPAR-γ was influenced by diabetes (Figure 2), suggesting that atheromas from these patients might respond differently. Our cell culture studies suggest that the PPAR-γ ligand rosiglitazone increased secretion of TFPI from HAECs. In contrast, Western blotting suggested that rosiglitazone had the unfavorable effect of reducing atheroma TFPI expression. This further suggests that it is difficult to model the complex responses of human atheromas in cell culture studies (Figure 4). In any case, we were unable to demonstrate any significant TFPI activity even in untreated atheromas, suggesting that any effect of rosiglitazone on TFPI action may be clinically irrelevant.

Carotid endarterectomy has been clearly shown to reduce the risk of subsequent stroke in patients with a recent TIA associated with a tight carotid stenosis; however, population studies suggest that a significant number of patients experience strokes while awaiting revascularization after the initial consultation. We therefore sought to use cell and explant cultures to investigate the potential of medication to acutely reduce thromboembolic events. The ability of cell culture to model the environment of the human atheroma is limited owing to the complex nature of the cellular and cytokine interactions in vivo. For example, in cell culture, TF must be upregulated in endothelial cells by added cytokines, whereas this is already the case in atheromas in vivo. Similarly, explant culture can only give a guide to the response of the atheroma over the short term in vivo, which is the period of interest in patients with symptomatic carotid atheromas. The heterogeneous nature of atheromas complicates drug incubation studies of this type. We therefore used paired, targeted biopsy specimens from the macroscopically diseased PICA and CCA. This approach controlled for patient difference, but even adjacent biopsy specimens from a macroscopically similar site may differ. In preliminary studies, we found that the coefficient of variation for TF in paired biopsy specimens was low at <5%. The inclusion of samples from patients who were receiving similar medication (all were prescribed statins and antiplatelet drugs) and the repetition of studies in 144 biopsy specimens support the validity of our findings. We thought that this was the most appropriate way to establish a medication for human trials of acute plaque stabilization. Another alternative would have been to study an animal model. However, the complex nature of human advanced unstable carotid atheromas is difficult to replicate, even in knockout mouse models. A number of the cytokine changes that are seen in human atherosclerosis are not demonstrated in mouse models.

Two recent trials examined the effects of fenofibrate and pioglitazone on cardiovascular events in patients with diabetes, although both studies were not designed to examine stroke as a primary outcome and did not specifically include patients with significant or symptomatic carotid atherosclerosis. The FIELD study, in which 9795 patients were randomized between the PPAR-α ligand fenofibrate and placebo, reported a reduction in total cardiovascular events but not stroke, although the results were confounded by the fact that more than twice as many patients in the placebo group commenced statin therapy. The PROACTIVE study, which randomized 5238 patients between the PPAR-γ ligand...
pioglitazone and placebo, recently reported a reduction in stroke rate in the treated patients. We were unable to demonstrate any beneficial effects of pioglitazone on carotid atheroma TF/TFPI in the acute setting (Figures 3 and 4).

In conclusion, our findings support the value of fenofibrate and rosiglitazone as agents for acute plaque stabilization in recently symptomatic patients with significant carotid artery narrowing. Further in vivo studies are required that will incorporate surrogate end points, such as cerebral microembolization identified on transcranial Doppler, to establish how clinically valuable these agents are. To clearly demonstrate a beneficial effect of these medications on the outcome of carotid atheromas, a sufficiently powered, randomized trial would be required to assess clinical end points, such as ipsilateral stroke, in patients awaiting carotid revascularization or in those thought to be unsuitable for intervention.

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This project was supported by a grant-in-aid from the National Heart Foundation; a cardiovascular lipid research grant; and the Townsville Stroke Research Fund.

Disclosures
None.

References
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