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

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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;38:000-000.)

Key Words: atherosclerosis, symptomatic • carotid artery • tissue factor

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fter a transient ischemic attack (TIA) or minor stroke, the risk of another significant neurologic event is particularly high in patients with a tight carotid stenosis.1-3 The main risk comes within the first few weeks after the initial neurologic event, with up to 20% of patients with a ≥50% carotid stenosis experiencing a stroke within 2 weeks.4 Carotid endarterectomy has been demonstrated to be effective in reducing stroke risk in subsets of symptomatic patients with carotid artery disease; however, the benefit of surgery declines rapidly with increasing time from the last ischemic event.5 For patients with a ≥50% carotid stenosis, the number of patients needed to undergo surgery to prevent 1 ipsilateral stroke was 5 for those randomized to treatment within 2 weeks and 125 for those randomized after >12 weeks.5 Population studies suggest that carotid revascularization is frequently not performed within this time window, and it may be difficult to achieve this treatment urgency in many public healthcare systems.4 The availability of medication that acutely reduces the stroke risk in this patient group and could be prescribed at the first consultation would be of significant value.

Histologic studies of excised carotid atherosclerotic material suggest that fibrous cap rupture plays an important role in cerebral ischemic stroke.1,6 Studies of patients experiencing cerebral ischemic events have identified particulate emboli within the middle cerebral artery, suggesting that thrombus generated at the site of carotid plaque atherosclerotic inflammation or ulceration later embolizes.7,8 The degree of thrombosis stimulated locally after plaque rupture is likely to be an important determinant of the clinical outcome. Tissue factor (TF) is the key initiator of the clotting cascade.9 After plaque...
rupture, exposure of TF leads to formation of the TF-factor VII (FVII)/FVIIa complex that drives generation of FXa and ultimately, thrombosis.9 The primary inhibitor of TF is tissue factor pathway inhibitor (TFPI), which complexes with FXa-TF/FVIIa to prevent further generation of FXa.10 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.11 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.12,13

Specimens

A conventional endarterectomy was performed, and specimens were immediately placed in cold culture medium and brought to the laboratory for experimental work.12 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.12 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...
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 8 days in culture,
explants were stored 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 passed
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 incu-
bated 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 immuno-
peroxidase method with antibody titers optimized in preliminary studies
to CD31 (Dako, JC70A, 4.5 μg/mL), α-smooth muscle 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
Diagnostic, TFPI0H10, 5 μg/mL), and TFPI (American Diagnostic, 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
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, TFPI0H10, 0.5 μg/mL; TFPI,
American Diagnostica, 4903, 1 μg/mL) and blotting condi-
tions 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 (asym-
ptomatic, nondiabetic, or untreated).12

TF and TFPI Activity
TF activity was assessed in cell culture with a commercial chromo-
genesis 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/FVIIa (American Diagnostic).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/hosome 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 proteinase 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 isof orm 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-γ1 and -γ2 in diabetic patients without symptoms (eg, for 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 fenofibrate (116.8±5.9, P=0.005), gemfibrozil (mean density units 79075±1105 compared with control 12 496±2349, P=0.005), gemfibrozil (mean density units 6949±1671 compared with control 9589±1938, P=0.04), and rosiglitazone (mean density units 53 500±19 792 compared with control 60 570±19 898, P=0.02) but not pioglitazone (mean density units 43 66±736 compared with control 4044±393, P=0.43) reduced TF 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 proteases 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 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 unstable 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.\(^7\) 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.\(^7,8\) 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 point, such as ipsilateral stroke, in patients awaiting carotid revascularization or in those thought to be unsuitable for intervention.

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**Disclosures**

None.

**References**

Figure I. Example of zymogram from explants incubated with PPAR ligands. Lanes 1, 2, and 3 were proteins extracted from conditioned media removed from a control explant at 24, 72, and 120 hours. Lanes 1, 2, and 3 were proteins extracted from conditioned media removed from a fenofibrate-treated explant at 24, 72, and 120 hours.