Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, prevents hypertensive vascular hypertrophy and fibrosis

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Submitted 2 September 2010; accepted in final form 6 December 2010

Gelosa P, Sevin G, Pignieri A, Budelli S, Castiglioni L, Blanc-Guillemaud V, Lerond L, Tremoli E, Sironi L. Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, on aorta remodeling were evaluated in spontaneously hypertensive stroke-prone rats (SHRSPs), a model of severe hypertension, endothelial dysfunction, vascular inflammation, and cerebrovascular diseases. Male SHRSPs were allocated to three groups receiving a standard diet (n = 5) or a high-sodium permissive diet plus vehicle (n = 6) or plus terutroban (30 mg·kg⁻¹·day⁻¹; n = 6). After 6 wk of dietary treatment, all of the animals were injected with bromodeoxyuridine and simultaneously treated with an antithrombotic agent. The effects of terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, on aorta remodeling were evaluated in spontaneously hypertensive stroke-prone rats (SHRSPs), an animal model that spontaneously develops proteinuria, severe hypertension, and cerebrovascular damage (31). The occurrence of brain abnormalities in this rat strain is invariably preceded by systemic inflammation, alterations in vasculature-bound barriers, endothelial dysfunction, and increased expression of endothelial adhesion molecules (1, 32). These features make SHRSPs particularly suitable for investigating the vascular effects of terutroban.

METHODS

Experimental plan. The procedures concerning animal care, surgery, and euthanasia were carried out in accordance with national and international laws and policies and approved and authorized by the Ministry of Health-University of Milan Committee (approval number: 1242003-A 13/10/2003). The experiments involved male SHRSPs purchased from Charles River (Calco, Lecco, Italy). Baseline measurements were made in all rats at 6–8 wk of age, after which the animals were randomized into three groups: one was fed a standard diet (n = 5), whereas the others (n = 6 each) were switched to a high-sodium diet (1% NaCl in drinking water). The Japanese permissive diet, containing 18.7% protein, 0.63% potassium, and 0.37% sodium (Laboratorio Dottori Piccioni, Gessate, Italy), and received 1% NaCl in drinking water. The Japanese permissive diet was chosen on the basis of preliminary experiments aimed at identifying its ability to delay the appearance of spontaneous brain damage and prolong survival. The food and liquid intake, blood pressure, body endogenous prostanooids agonists of TPα generated from the nonenzymatic oxidation of arachidonic acid by means of a free radical-catalyzed mechanism (23). Like TPα ligands, IPs promote platelet activation and TXA2 formation and have a number of other biological effects such as increasing the expression of inflammatory cytokines and matrix metalloproteases, increasing endothelin-1 levels, all of which are involved in athrogenesis (10). In light of these findings, several TPα antagonists have recently been synthesized and their protective effect(s) in vascular diseases has been widely described in experimental studies (16).

Terutroban is a selective TPα antagonist with antithrombotic, antivasoconstricting, and antiatherosclerotic properties (7) that was developed for the secondary prevention of cardiovascular events in patients with a history of ischemic stroke (4, 5). A number of in vivo experimental studies have found that terutroban prevents vascular wall proliferation (8) and athrogenesis in mice (6), as well as in a rabbit model of atherosclerosis and restenosis (37). In the present study, we explored its effects on arterial remodeling in spontaneously hypertensive stroke-prone rats (SHRSPs), a model that spontaneously develops proteinuria, severe hypertension, and cerebrovascular damage (31). The occurrence of brain abnormalities in this rat strain is invariably preceded by systemic inflammation, alterations in vasculature-bound barriers, endothelial dysfunction, and increased expression of endothelial adhesion molecules (1, 32). These features make SHRSPs particularly suitable for investigating the vascular effects of terutroban.
weight, and proteinuria of the animals were followed weekly as previously described (31).

After 6 wk of dietary treatment, all of the animals were injected twice a day for 2 days with 50 mg/kg of bromodeoxyuridine (BrdU) dissolved in saline and were euthanized 2 h after the last injection. The thoracic aorta was collected and rinsed in ice-cold PBS to clean adventitial fat, after which the connective tissue was removed by blunt dissection and the aorta was cut into two halves: the upper half was frozen in cold isopentane and stored at −80°C until use, whereas the lower half was fixed in Carnoy reagent (Merck, Darmstadt, Germany) and embedded in paraffin (Sigma-Aldrich, St. Louis, MO).

**Immunohistochemistry.** Paraffin-embedded aorta sections (8 mm) were dewaxed in xylene, hydrated, and then permeabilized in 10 mM sodium citrate and 0.05% Tween-20 (pH 6) for 20 min at 90°C. For heat shock protein (HSP)-47 detection, endogenous peroxidase was blocked by adding 1% H2O2 in 1:1 methanol-PBS and nonspecific binding sites were saturated with 10% normal goat serum. The sections were incubated overnight at 4°C with the primary mouse antibody anti-HSP47 (1:200; StressGen, Ann Arbor, MI) and stained for horseradish peroxidase immunohistochemistry. For fluorescence immunohistochemistry, after the nonspecific binding sites were blocked with 10% goat serum, the sections were incubated overnight at 4°C with the primary mouse antibody anti-vimentin (1:200; Dako, Glostrup, Denmark), anti-proliferating cell nuclear antigen (PCNA; 1:200; DAKO), anti-α-smooth muscle actin (1:250; Sigma-Aldrich), and rabbit anti-fibronectin antibody (1:200; Dako). Antigen retrieval for BrdU labeling was performed by means of permeabilization in 2 M HCl for 30 min at 37°C before the addition of the primary antibody (rat anti-BrdU, 1:150; Abcam, Cambridge, MA). Detection was performed using specific goat secondary antibodies conjugated with Alexa Fluor 555 (1:600; Molecular Probes/Invitrogen) or Alexa Fluor 488 (1:400; Molecular Probes/Invitrogen) for 2 h at room temperature and with Hoechst 33258 nuclear staining (2.5 g/ml; Invitrogen) for 15 min. Immunofluorescence detection of cleaved caspase-3 (1:200; Cell Signaling) was performed following the standard protocol of the Tyramide signal amplification fluorescence system after quenching the endogenous peroxide. The presence of apoptotic cells in the vessels was also investigated using an ApoAlert DNA fragmentation assay kit in accordance with the data sheet protocol (Clontech, Mountain View, CA).

The negative control did not show any nonspecific staining. The fluorescence images were acquired using an inverted microscope (Axiovert 200, Zeiss) and the supplied hardware and software (Axiovision, Zeiss) and fixed exposure times for comparisons.

**Histological analysis.** Deparaffinized 8-μm-thick aorta sections were stained with standard hematoxylin-eosin and examined by light microscopy. To visualize collagen fibrils, Sirius red F3BA (0.5% in saturated aqueous picric acid) was used for 40 min. The slices were viewed using polarized light microscopy (Axiovert S100 TV microscope; Zeiss, Jena, Germany) to detect birefringent collagen fibrils.

**Image analysis.** The coronal views of the aorta slices (each area 650 mm × 870 mm) were combined to obtain a total aorta section of each animal. Computer-assisted image analysis (ImageJ; OPTIMAS 6.2; Media Cybernetics) was used to analyze the wall thickness-to-lumen ratio (the media surface area to the inner vessel surface area), total collagen deposition (the calculated ratio between the surface area positively stained with Sirius red and the media surface area), fibronecin deposition (positive area/media surface area), and the percentage of HSP47 (the ratio between the HSP47+ media surface area and the media surface area). The number of BrdU+ or PCNA+ cells refers to the number of positive cells in a single aorta section. The values are expressed as means ± SE.

Three sections 2 mm apart were analyzed for each animal by the same pathologist, who was unaware of the nature of the experimental groups.

**Analysis of mRNA for TGF-1β.** Total RNA was prepared by guanidium thiocyanate denaturation from frozen aorta collected from the rats receiving a standard diet and from the vehicle- and terutroban-treated rats (n = 5 rats each group). The expression of TGF-1β was measured by means of semiquantitative RT-PCR as previously described by Sironi et al. (32). GAPDH was amplified as a standard. The RT-PCR products were separated on 1.5% agarose gel and visualized using GelRed nucleic acid gel stain (Biotium). The intensity of each band was quantified using National Institutes of Health Image software and expressed in arbitrary units. The densities of the TGF-1β bands were normalized using the corresponding GAPDH signal.

**Statistical analysis.** The data were statistically compared using one-way ANOVA and unpaired t-tests. The data are expressed as mean values ± SD, and a P value of <0.05 was considered significant.

**RESULTS**

**Effects of terutroban on blood pressure, body weight, and proteinuria.** Baseline mean systolic blood pressure in the SHRSPs was 170 ± 20 mmHg. Throughout the treatment period, the animals treated with the standard diet showed only a slight increase in blood pressure (~10 mmHg), whereas after 6 wk of high-salt dietary treatment, blood pressure progressively increased to 250 ± 24 in the vehicle-treated rats and 230 ± 15 mmHg in the terutroban-treated rats. Terutroban had no significant effect on food, fluid intake, or body weight (data not shown). At the beginning of the dietary treatment, the level of proteinuria in the SHRSPs as a whole was <10 mg/day; after 6 wk, it increased to an average of 240 ± 40 mg/day in the vehicle-treated rats but was significantly lower in the rats treated with terutroban (40 ± 10 mg/day; P < 0.001 vs. vehicle). The SHRSPs receiving the standard diet maintained a mean proteinuria level of 10 ± 4 mg/day (P < 0.01 vs. vehicle and vs. terutroban).

**Terutroban prevents increased aortic thickening in salt-loaded SHRSPs.** Hematostatin-eosin staining of cross sections of SHRSP aortas collected after 6 wk of dietary treatment indicated that the media thickness-to-lumen ratio was significantly higher in the salt-loaded rats than in those fed the standard diet (0.39 ± 0.1 vs. 0.17 ± 0.07; P < 0.001) (Fig. 1), but treatment with terutroban completely prevented the increase (0.16 ± 0.07; P < 0.001 vs. vehicle) (Fig. 1).

**Effects of terutroban on the proliferation of vascular cells and vimentin expression.** Aortic rings harvested after 6 wk of dietary treatment underwent BrdU immunohistochemical detection to assess the cell proliferation index.

As shown in Fig. 2, A–C, the number of BrdU-positive cells was higher in the vehicle-treated rats receiving the high-salt diet than in the rats fed the standard diet (46.7 ± 2.65 vs. 5.83 ± 1.01; P < 0.0001) but was significantly lower in the terutroban-treated rats (14.7 ± 3.75; P < 0.0001). Most of the BrdU+ cells were located in the media and also positively stained for α-smooth muscle actin, a selective marker of SMCs (Fig. 2, D–F). A few BrdU+ cells were also detectable in the intima and adventitia (Fig. 2E).

PCNA staining revealed a dramatic increase in the number of positive cells in the media of salt-loaded SHRSPs treated with vehicle compared with the rats fed the standard diet (60.7 ± 14.7 vs. 12.5 ± 0.5; P < 0.0001), but terutroban completely prevented this increase (19.5 ± 9.5; P < 0.0001). There were also more PCNA-positive cells in the intima of the salt-loaded rats receiving vehicle (21.3 ± 11.8) than in the intima of the rats fed the standard diet (0.09 ± 0.08; P <
Neither immunohistochemical analysis of cleaved caspase-3 nor DNA fragmentation revealed the presence of apoptotic cells in any of the aortic rings from the animals in the different experimental groups (data not shown).

Furthermore, the expression of the cytoskeletal protein vimentin in the intima and inner media of the vehicle-treated rats was higher than in the rats fed the standard diet or those treated with terutroban, in which vimentin was absent or only barely detectable (Fig. 3, A–C).

**Effects of terutroban on vascular fibrosis.** The Sirius red-stained aorta sections from all of the experimental groups showed yellow/red birefringent collagen fibers under polarized light, mainly in the adventitia but also, although to a lesser extent, in the tunica media (Fig. 3, D–F). The area positive for interstitial fibrillar collagen was significantly larger in the vascular wall of the aortic rings from the vehicle-treated animals than in those from the standard diet group (0.41 ± 0.02 vs. 0.31 ± 0.01; \( P < 0.01 \)); once again, terutroban treatment significantly prevented this increase (0.32 ± 0.02; \( P < 0.001 \)) (Fig. 3, D–F).

Fibronectin appeared to be diffused in the extracellular matrix of the media of all of the experimental groups, and this was more marked in the adventitia. As in the case of collagen, positive fibronectin labeling was higher in the vehicle-treated group than in the rats fed the standard diet (0.61 ± 0.02 vs. 0.42 ± 0.03; \( P < 0.01 \)) or those treated with terutroban (0.32 ± 0.04; \( P < 0.01 \)) (Fig. 3, G–I).

**Terutroban inhibits HSP47 and TGF-1 expression.** HSP47 was immunohistochemically detectable in the SMCs of the adventitia and media of the aorta of the SHRSPs (Fig. 4 A). Cell
staining was significantly greater in the media of the vehicle-treated rats than in that of the rats fed the standard diet (0.61 ± 0.05 vs. 0.53 ± 0.03; P < 0.01). Terutroban treatment not only completely prevented this increase but also reduced HSP47 levels to below those observed in the rats fed the standard diet (0.32 ± 0.02; P < 0.01 vs. vehicle) (Fig. 4B).

The expression of TGF-β1 mRNA was slightly more marked in the aortas of the vehicle-treated rats than in those of the rats fed the standard diet (P < 0.01); terutroban treatment prevented this increase in mRNA expression (P < 0.001), which remained at a slightly lower level than in the rats fed the standard diet (Fig. 4C).

**DISCUSSION**

In addition to inflammation, vascular remodeling accompanies a number of pathological situations, including atherosclerosis, hypertension, and restenosis. Vascular remodeling can affect every layer of the arterial wall and may include neointimal hyperplasia, medial hyper- or hypotrophy, and adventitial fibrosis, all of which lead to changes in vessel size and luminal width (35). In particular, medial thickening is mainly characterized by the accumulation of SMCs and extracellular matrix proteins.

Prostanoids are major contributors to the pathogenesis of chronic diseases, particularly in relation to vessel inflammation, and thus have a direct impact on the cardiovascular system (10). By binding to their vascular receptors, TXA2 and IPs play a key role in modulating inflammatory processes and directly affect the vascular phenotype by means of a variety of mechanisms (23). The pharmacological modulation of TPr activation is therefore a relevant target for the prevention or treatment of vascular disease (10, 15). Clinical and experimental observations indicate that terutroban, a specific TPr antagonist, is a particularly promising means of treating atherothrombosis and preventing or delaying atherogenesis (7, 23).

This study explored the ability of terutroban to modulate the vascular remodeling that naturally occurs in the thoracic aorta of SHRSPs. Previously published data obtained in the same rat strain show that chronic terutroban treatment increases survival by reducing systemic inflammation and improving vascular reactivity in isolated carotid artery (11). The results of the present study show that chronic terutroban treatment also inhibits vascular hypertrophy and fibrosis in salt-loaded SHRSPs.

Specifically, aortic rings collected from vehicle-treated rats after 6 wk of high-salt dietary treatment showed a significant increase in aortic wall thickening compared with rats fed the standard diet. This is accompanied by the increased proliferation of SMCs documented by the larger number of cells incorporating BrdU or by increased media immunoreactivity to PCNA, a nuclear protein that is important for the progression of the G1 phase (33). These effects were prevented by the
chronic administration of terutroban, which is in line with previously published data indicating that TPr deletion or its antagonism by terutroban is efficacious in preventing changes in the intima-to-media ratio, percent stenosis, and the incorporation of BrdU in the arteries of mice undergoing vascular injury (8).

These findings are in line with in vitro data indicating the abnormal proliferation of primary cultured aortic VSMCs isolated from SHRSPs compared with the normotensive Wistar-Kyoto reference strain (9), which is also associated with greater susceptibility to death by apoptosis. However, in the mentioned in vitro study, apoptosis was only recorded after serum mitogens and survival factors had been completely withdrawn, and this was prevented by the addition of serum to the cell medium (a situation that is closer to our in vivo approach).

Under our conditions, DNA fragmentation assay and caspase-3 staining did not reveal any detectable signs of apoptosis. These data are particularly interesting because the remodeled vessel wall is characterized by proliferative and apoptosis-resistant VSMCs in many situations such as vascular restenosis after injury. In experimental models of in vivo rat carotid balloon injury and ex vivo human saphenous vein grafts in tissue culture, wall thickening is related to SMC proliferation (as shown by the increase in the number of PCNA+ cells), whereas the caspases involved in apoptosis are inactivated (2).

The remodeled pulmonary arteries associated with pulmonary arterial hypertension are also characterized by suppressed apoptosis, vasoconstriction, and increased proliferation within the vascular wall (19).

An increase in the expression of vimentin, an intermediate filament protein of the cytoskeleton of vascular endothelial cells, is also found in the proliferative arterial cells of SHRSPs (13). Some authors have suggested that increased vimentin is an acute endothelial responsiveness, especially when the stimulus is a mechanical factor such as the shear stress occurring during hypertension (18, 29). Moreover, when exposed to dynamic stimuli or natural aging, cells other than endothelial cells (including VSMCs in the media area) significantly express vimentin (21). The vimentin network is important in stabilizing cell shape and opposing remodeling (30). The vimentin network is also involved in the progression of vascular neointimal formation, which supports the hypothesis that HSP47 plays a crucial role in SMC collagen metabolism.

Previous in vitro and in vivo studies have convincingly shown that HSP47 plays an important role in the onset and progression of various fibrotic diseases, particularly those affecting the artery wall (24). HSP47 is localized in the endoplasmic reticulum of collagen-producing cells and is thought to be essential for the synthesis, processing, and secretion of procollagen molecules (17). It is also expressed by human arterial SMCs in culture and has been identified in the fibrous cap of human atherosclerotic plaque (25, 26). It is therefore likely that the similar coexpression of HSP47 and collagen is upregulated during the progression of vascular neointimal formation, which supports the hypothesis that HSP47 plays a crucial role in SMC collagen metabolism.

Furthermore, the expression of HSP47 is modulated by TGF-β, one of the principal fibrinogenetic cytokines (3) that stimulates cell hypertrophy and extracellular matrix production (20) and plays a key role in in vivo vascular remodeling (36).
It also induces tissue fibrosis by activating HSF1, a ubiquitous transcription factor for various HSPs. HSF1 stimulates the transcription of the expression of HSP47 mRNA and leads to the increased expression of HSP47 protein (27). As HSP47 acts as a molecular chaperone for all types of collagen, its inhibition may be a means of preventing or attenuating fibrosis. Our data indicate that chronically treating salt-loaded SHRSPs with terutroban significantly prevents both TGF-β1 and HSP47 expression and consequently has beneficial effects on the development of fibrotic processes in rat aortic wall.

In conclusion, our findings indicate that terutroban prevents the development of hyperplasia in the aorta of hypertensive rats and has a beneficial effect on fibrotic processes without affecting blood pressure. These effects are at least partially due to its effect on TGF-β1 and, consequently, HSP47 expression. Our findings suggest that TP receptor activation plays an important role in the studied processes and provides mechanistic evidence explaining the beneficial effects of terutroban in preventing or delaying atherogenesis.

REFERENCES

GRANTS

This work was supported by Institut de Recherches Internationales Servier, Courbevoie Cedex, France.

DISCLOSURES

V. Blanc-Guillemaud and L. Lerond are employees of Servier.

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before cerebral ischemia in stroke-prone rats; identification by proteomics. 


