Gliotoxin Inhibits Neointimal Hyperplasia after Vascular Injury in Rats

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Key Words

Carotid artery · Cytoskeleton · Migration of cells · Neointimal formation · Proliferation of cells · Vascular smooth muscle cells

Abstract

Neointima formation participates in the pathophysiology of atherosclerosis and restenosis. Proliferation and migration of vascular smooth muscle cells (VSMC) are initial responses to vascular injury. The aim of the present study was to assess the effect of gliotoxin, an inhibitor of nuclear factor (NF)- κ B, on migration and proliferation of cultured rat VSMC and neointimal formation in injured rat vessels. In cultured VSMC, gliotoxin inhibited the nuclear translocation of the p65 subunit of NF- κ B in response to inflammatory stimuli. In addition, gliotoxin inhibited VSMC migration and proliferation in response to platelet-derived growth factor-BB. This was associated with a rapid rearrangement of the F-actin and vimentin cytoskeleton. Furthermore, gliotoxin inhibited endothelial cell nuclear translocation of p65, cell surface expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, and monocytic cell adhesion to a cytokine-activated endothelial monolayer. In the rat carotid artery balloon catheter injury model, the systemic administration of gliotoxin for 10 days decreased neointimal hyperplasia and luminal stenosis by up to 90% and decreased the expression of proliferating cell nuclear antigen in the vessel wall by up to 70%, depending on the dose. These observations suggest

that gliotoxin favorably regulates the response to vascular injury through actions on VSMC. However, further studies evaluating the therapeutic benefit of gliotoxin in restenosis after balloon angioplasty are required.

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Introduction

Neointimal hyperplasia has been identified as one of the mechanisms of arterial restenosis after balloon angioplasty in humans. The high frequency of restenosis (30–50% of patients) limited the long-term success of percutaneous transluminal coronary techniques [1–3]. The use of drug-eluting stents has greatly reduced the incidence of restenosis [4–7]. However, recent reports suggest that sirolimus- or paclitaxel-eluting stents are associated with an increased risk of late stent thrombosis, a potentially fatal complication, when compared with bare-metal stents [8–13]. Thus, the search for alternative agents would be desirable. Gliotoxin belongs to the epipolythiodioxopiperazine class of biologically active secondary fungal metabolites [14]. It is characterized by the presence of a quinoid moiety and a disulfide bridge across the piperazine ring that is essential for its activity. Gliotoxin exhibits profound immunosuppressive effects both in vitro and in vivo, inhibiting activation and proliferation of

¹ Deceased.

B and T lymphocytes [15–18]. In addition, gliotoxin inhibits the activation of the transcription factor nuclear factor (NF)- κ B [17, 19, 20] and has proven safe and effective in controlling inflammation in animal models [21].

Arterial wall injury leads to intimal thickening and luminal stenosis, with vascular smooth muscle cells (VSMC) as key players [22–24]. Endothelial denudation of the rat carotid artery by angioplasty has provided a rapid, highly reproducible and widely used model to study cellular and molecular events in VSMC leading to neointimal formation and restenosis [3, 25–27].

The main purpose of the current study was to evaluate the effect of gliotoxin on the inhibition of neointimal hyperplasia in a rat model of vascular injury induced by balloon angioplasty and to explore the possible contribution of direct actions on VSMC, investigating the potential role of gliotoxin therapy in the prevention of restenosis.

Materials and Methods

Cell Cultures

Thoracic aortas from Sprague-Dawley rats were removed and stripped of endothelium and adventitia. The vessels were cut into small segments. VSMC were isolated by collagenase type II (Sigma) enzymatic digestion and the explant method [28]. Cell cultures were maintained at 37°C in 5% CO₂ in Dulbecco's modified minimal essential media (DMEM; Gibco), supplemented with 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin and fungizone. Cells from passages 3–8 were studied. Purity was 98%, as assessed by positive immunostaining with anti-smooth muscle α -actin monoclonal antibody (Sigma).

Human umbilical vein endothelial cells were cultured as described elsewhere [29]. Briefly, umbilical veins were cannulated and perfused with M199 culture medium to remove blood, and then incubated with 0.1% type P collagenase (Roche Diagnostics) for 20 min at 37°C. After removal of collagenase, cells were harvested and cultured in M199 medium (BioWittaker) with 20% heat-inactivated FCS and antibiotics. Confluent cells between the first and fourth passage were studied. Cells were maintained in M199 medium with 10% FCS, 50 μ g/ml endothelial cell growth supplement, 100 μ g/ml heparin (Sigma) and antibiotics. The purity of endothelial cell cultures was characterized by the typical cobblestone morphology and >98% positive staining for von Willebrand's factor.

Human monocytic THP-1 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (Gibco) with 10% FCS and antibiotics.

Cell Migration Assays

Rat VSMC chemotaxis was studied using Transwell® inserts with polycarbonate membranes (8- μ m pore size; Costar). Cells were suspended at 5×10^5 /ml in DMEM containing 0.5% BSA (DMEM-BSA), added to the upper chamber in a final volume of 100 μ l and treated with gliotoxin (Sigma). Chemotaxis chambers were assembled by adding 50 ng/ml platelet-derived growth fac-

tor (PDGF)-BB (PeproTech) in DMEM-BSA to the lower compartments in a final volume of 600 μ l. After 18 h of incubation at 37°C, the cells on the upper surface were mechanically removed, and cells remaining on the underside of the filters were fixed in 2% paraformaldehyde and stained with 0.5% crystal violet. The color was eluted with 10% acetic acid, and measured at 600 nm in a spectrophotometer. Specific chemotaxis was expressed as the percentage of VSMC that migrated in response to PDGF-BB without gliotoxin. All data points were assessed in duplicate for each condition.

Immunofluorescence

Cytoskeletal protein organization, proliferating cell nuclear antigen (PCNA) expression and nuclear translocation of NF- κ B p65 were assessed by immunofluorescence in subconfluent cells cultured on 8-well glass chamber slides (Lab-Tek; Nalge Nunc).

To assess cytoskeletal proteins, cells were incubated with 100 ng/ml gliotoxin for 1 h, fixed in 2% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. F-actin was stained with 800 μ M FITC-labeled phalloidin (Sigma) for 30 min at room temperature. Vimentin and β -tubulin were stained with specific monoclonal antibodies on ice for 45 min (Sigma), followed by an FITC anti-mouse IgG (Sigma). After rinsing with PBS, samples were mounted with 90% buffered glycerin. Immunofluorescence was determined using a Nikon Eclipse E400 microscope and photographed with a Nikon Coolpix 990 digital camera.

To assess PCNA expression, cells were growth arrested in serum-free medium for 48 h before preincubation with 100 ng/ml gliotoxin for 90 min and subsequent stimulation with DMEM/10% FCS or 10 ng/ml PDGF-BB for 24 h. Cells were fixed and permeabilized with methanol/acetone (1:1) at 4°C for 15 min. Immunostaining was performed with anti-PCNA antibody clone PC10 (Sigma) at 4°C for 24 h; then an FITC goat anti-mouse IgG was added for 45 min at 4°C.

To assess nuclear translocation of p65, cells preincubated with 100 ng/ml gliotoxin for 1 h were treated with 30 ng/ml IL-1 β (PeproTech) for 2.5 h, and then fixed and permeabilized with paraformaldehyde and Triton X-100. Primary antibody was rabbit polyclonal anti-p65 (Santa Cruz Biotechnology) at 37°C for 1 h. Then FITC-goat anti-rabbit IgG (Sigma) was added for 45 min at room temperature.

Monocytic Cell Adhesion to Endothelial Cells

For cell adhesion studies, 10⁴ endothelial cells were seeded in gelatin-coated 96-well plates (Costar), grown to confluence and pretreated with gliotoxin for 1 h in M199 containing 0.5% BSA (M199-BSA). Subsequently, cells were stimulated with 50 ng/ml IL-1 β (Immugenex) at 37°C for 4 or 24 h. After washing, 100 μ l of 2×10^6 THP-1 cells/ml in RPMI-BSA, labeled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes), were added to each well. THP-1 cells were allowed to adhere to the endothelial monolayer for 1 h at 37°C, and non-adherent cells were washed off with six rounds of fresh medium. The residual adherent cells were quantified in a spectrofluorometer (Luminescence Spectrometer LS50B; Perkin Elmer). All assays were performed in triplicate, and the percentage of adhesion was referred to IL-1 β -activated endothelial cells in the absence of inhibitors, after subtraction of adherence to untreated cells.

Flow Cytometry

Cell surface expression of VCAM-1, ICAM-1 and E-selectin was analyzed by flow cytometry. Confluent endothelial cells were activated with 50 ng/ml IL-1 β for 4 or 24 h. Cells were detached with Versene (EDTA) solution (BioWittaker) and stained with monoclonal antibodies to VCAM-1/CD106 (clone 1.G11B1), ICAM-1/CD54 (clone P2A4) and E-selectin/CD62E (clone P2H3; Endogen) for 30 min at 4°C. The secondary antibody was FITC goat anti-mouse IgG (Sigma). Negative controls were prepared by omitting the first specific antibody. Cells were fixed in 1% paraformaldehyde in PBS and fluorescence quantified in an EPICS XL-MCL flow cytometer.

Vascular Injury in Rat Carotid Artery

Male Sprague-Dawley rats (450–500 g) were anesthetized by intramuscular administration of ketamine (Ketolar; Parke Davis) and xylazine (Rompun; Bayer). The left common carotid artery was denuded of endothelium with a 2-french Fogarty balloon angioplasty catheter (Baxter), leaving the uninjured right side as a control. The balloon was introduced through an incision in the external carotid artery down to the aortic arch, inflated with saline, retracted along the full length of the common carotid artery and then deflated. This procedure was repeated three times, with the catheter turned 90° each time while being retracted. After removal of the catheter, the left external carotid artery was permanently ligated. Eight rats were treated with 200 or 400 μ g gliotoxin/kg body weight/day i.p. 4 h after injury and daily for 10 days. The dose was chosen based on previous laboratory experience [21]. Gliotoxin was well tolerated, and no adverse effects were noted in terms of body weight or general well-being. All animal studies were performed according to protocols approved by the Animal Care and Use Committee of our Institution, following international regulations.

Tissue Processing and Quantitative Histomorphometric Analysis

Ten days after injury, the common carotid arteries were cleared of blood with saline, excised, immediately embedded in Tissue-Tek OCT medium (Sakura) and frozen. Using a cryostat (Leica; Leitz), arteries were cut in transverse rings (7- μ m sections) taken from the center portion of the vessels, spaced at 0.5-mm intervals and stained using hematoxylin and eosin. Neointimal thickness was determined by subtracting the area defined by the luminal surface from the area defined by the internal elastic lamina, and the medial thickness was determined by the area of the internal elastic lamina subtracted from the external elastic lamina, using a computer-based image analysis program (Cellular Analysis System; Becton Dickinson) and a Nikon scope. Morphometry was performed in at least five individual sections of each arterial segment, and measurements were averaged for statistical analysis to determine the intima/media ratio and luminal stenosis.

Immunohistochemistry

Proliferative activity was evaluated in cryostat sections of carotid arteries. Slides were pretreated with cold acetone for 10 min and methanol with 0.3% H₂O₂ for 30 min to block endogenous peroxidase. Samples were incubated with anti-PCNA clone PC10 (1:50) for 24 h at 4°C, followed by a horseradish peroxidase-conjugated IgG (Amersham Biosciences) diluted 1:200 for 1 h at 4°C.

Color was developed by a diaminobenzidine chromogen system (Dako). Samples were slightly counterstained with hematoxylin. Cells were considered positive for PCNA expression only in the presence of an intense brown staining of the nucleus.

Statistical Analysis

Results are presented as means $\pm$ SD. Statistical comparisons were analyzed by nonparametric Mann-Whitney test or ANOVA, and values of $p < 0.05$ were considered significant.

Results

Gliotoxin Inhibits PDGF-BB-Induced Migration of VSMC

We first addressed the influence of gliotoxin on the migration of rat VSMC in response to PDGF-BB, one of the most powerful chemoattractants for VSMC [30–32]. Gliotoxin abolished the chemotactic response of VSMCs to PDGF-BB in a time- and dose-dependent manner (fig. 1). Treatment of VSMC with 100 ng/ml gliotoxin for 1 h inhibited 31% of migration referred to the positive control (fig. 1a). Inhibition increased to 45% with 200 ng/ml gliotoxin (data not shown). An inhibition very close to basal levels was found after a 3-hour incubation with 200 ng/ml gliotoxin (fig. 1b). Gliotoxin was not cytotoxic for VSMC as assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reduction colorimetric assay [33].

Gliotoxin Rearranges the Actin and Vimentin Cytoskeleton of VSMC

We next studied possible mechanisms whereby gliotoxin abolished the migratory response of vascular cells. Cellular locomotion is mediated by coordinated changes in the assembly and disassembly of F-actin fibers [34, 35]. VSMC growing under control conditions showed normal appearance of the F-actin cytoskeleton containing numerous microfilaments organized in stress fibers, which traverse the length of the cells (fig. 2a). Incubation with 100 ng/ml gliotoxin for 1 h resulted in a complete disorganization of the F-actin cytoskeleton (fig. 2b). Vimentin formed a fine fibrillar network, filling almost the whole cytoplasm, and extended to the cell periphery in untreated cells (fig. 2c). After incubation with 100 ng/ml gliotoxin for 30 min, vimentin filaments redistributed and aggregated into dense structures at the perinuclear area (fig. 2d). By contrast, the basal distribution pattern of β -tubulin (fig. 2e), displaying a radial architecture of microtubules connected to the perinuclear region and reaching the plasma membrane, was barely modified by

Fig. 1. Gliotoxin inhibits VSMC migration in a time (a)- and dose-dependent (b) manner. Cells were seeded on the upper chamber of Transwell inserts, and chemotaxis was induced by 50 ng/ml PDGF-BB added to the lower compartment for 18 h. **a** VSMC were pretreated with 100 ng/ml gliotoxin for 1–3 h before the addition of PDGF-BB. Data are means $\pm$ SD from three independent experiments performed in duplicate. * $p < 0.005$; ** $p < 0.0001$, vs.

cell migration in response to PDGF-BB without gliotoxin. **b** VSMC were pretreated with serial concentrations of gliotoxin for 3 h before the addition of PDGF-BB. Results are means $\pm$ SD from four independent experiments performed in duplicate. * $p < 0.0001$; ** $p < 0.0009$; *** $p < 0.05$, vs. cell migration in response to PDGF-BB without gliotoxin.

Fig. 2. Gliotoxin rearranges the cytoskeletal architecture of actin and vimentin in VSMC. The distribution of cytoskeletal proteins was studied by immunofluorescence. Cells were examined under control conditions (a, c, e) or after treatment with 100 ng/ml gliotoxin (b, d, f) for 1 h. VSMC were stained with FITC-phalloidin for F-actin (a, b) and with antibodies against vimentin (c, d) and β -tubulin (e, f). Fluorescent micrographs are representative of at least three independent experiments. Original magnification, $\times 400$.

Color version available online

Fig. 3. Gliotoxin inhibits the expression of PCNA in VSMC. Quiescent cells were untreated (**a**, **c**, **e**) or preincubated with 100 ng/ml gliotoxin for 90 min (**b**, **d**, **f**), and then cultured in medium alone (**a**, **b**), stimulated with 10% FCS (**c**, **d**) or with 10 ng/ml PDGF-BB (**e**, **f**) for 24 h. Representative images of three independent experiments are shown. Original magnification, $\times 200$. **g** PCNA was quantified by counting positive nuclei (expressed as the percentage referred to total nuclei). Data are means $\pm$ SD from 6 independent experiments (10 fields for each condition per experiment). * $p < 0.023$; ** $p < 0.0001$, untreated cultures (■) vs. gliotoxin-treated groups (□).

incubation with gliotoxin (fig. 2f). Cell shape remained unchanged after treatments.

Gliotoxin Inhibits the Expression of PCNA in VSMC

Together with migration, proliferation also plays a key role in restenosis [36, 37]. PCNA is a marker of cells that are actively proliferating. We studied the effect of gliotoxin on VSMC proliferation induced by two potent mitogens for these cells, serum and PDGF-BB. Immunocytochemical analysis of quiescent cells showed a small number of PCNA-positive nuclei (fig. 3a), the staining being virtually absent after gliotoxin treatment (fig. 3b). Strong fluorescence was observed in most nuclei after in-

cubation for 24 h with both serum (fig. 3c) or PDGF-BB (fig. 3e). Pretreatment with gliotoxin decreased the number of PCNA-positive cells by 72% in the case of serum (fig. 3d) and by 66% for PDGF-BB (fig. 3f, quantified in fig. 3g).

Gliotoxin Inhibits the Nuclear Translocation of the NF- κ B p65 Subunit in VSMC

Nuclear translocation of p65 is necessary for its transcription factor activity. Inflammatory cytokines, such as IL-1 β , promote NF- κ B activation [38–40]. In untreated VSMC, p65 immunoreactivity is present in cytoplasm but absent from nuclei (fig. 4a). IL-1 β reduced cytoplas-

Fig. 4. Gliotoxin prevents the nuclear translocation of NF- κ B p65 in VSMC. Fluorescent micrographs show the cytosolic localization of p65 in untreated control cells (a) and the nuclear translocation following stimulation with 30 ng/ml IL-1 β for 2.5 h (b)

which was prevented by pretreatment with 100 ng/ml gliotoxin (c). Representative images of three independent experiments are shown. Original magnification, $\times 400$.

Fig. 5. Gliotoxin inhibits the adhesion of THP-1 monocytic cells to IL-1 β -activated endothelial cells. Endothelial cells treated with different concentrations of gliotoxin for 1 h were activated with 50 ng/ml IL-1 β for 4 (a) or 24 h (b). Adhesion of THP-1 cells was

evaluated in the last hour of the experiment. Adhesion in the absence of gliotoxin was considered to be 100%. Means $\pm$ SD of 6 experiments; * $p < 0.0001$, vs. 0 ng/ml gliotoxin.

mic staining and resulted in new nuclear staining, demonstrating translocation of the activated NF- κ B protein from the cytoplasm to the nucleus (fig. 4b). Pretreatment of cells with 100 ng/ml gliotoxin prevented the nuclear translocation of p65 induced by IL-1 β (fig. 4c), suggesting that gliotoxin inhibited the activation of this nuclear factor in rat VSMC.

Gliotoxin Inhibits Monocytic Cell Adhesion to Activated Endothelial Cells

Adhesion to an activated endothelium is the first step in leukocyte extravasation. Pretreatment of endothelial cells with gliotoxin for 1 h dose-dependently reduced

monocytic THP-1 cell adhesion to endothelial cells activated with IL-1 β for either 4 or 24 h (fig. 5). Gliotoxin was not cytotoxic for endothelial cells as assessed by the MTT assay. Gliotoxin prevented the nuclear translocation of p65 (RelA) induced by IL-1 β (fig. 6a) as well as cell surface expression of the NF κ B-dependent adhesion molecules VCAM-1, ICAM-1 and E-selectin (fig. 6b–d).

Gliotoxin Inhibits Neointimal Formation in a Rat Carotid Artery Model of Vascular Injury

To determine directly whether the in vitro effects of gliotoxin were relevant in vivo, we used the well-established rat carotid injury model to examine its inhibition

Fig. 6. Gliotoxin prevents NF- κ B activation and reduces adhesion molecule expression induced by IL-1 β in endothelial cells. **a** Pre-incubation with gliotoxin (100 ng/ml for 30 min) prevents p65 translocation from the cytosol to the nucleus induced by stimulation with 10 ng/ml IL-1 β for 60 min. Magnification: $\times 400$. **b–d** Representative flow-cytometric diagrams showing that pre-

treatment with gliotoxin decreases cell surface expression of ICAM-1 (**b**), VCAM-1 (**c**) and E-selectin (**d**) induced by stimulation with 50 ng/ml IL-1 β for 4 (**d**) or 24 h (**b**, **c**). Negative control cells were stained with the secondary antibody. They are not presented for ICAM-1 since it overlaps with basal expression. Representative of 6 independent experiments.

potential on neointima formation. Uninjured normal carotid arteries presented the endothelial monolayer (fig. 7a). Neointimal hyperplasia dramatically developed within 10 days after angioplasty injury (fig. 7b). Gliotoxin inhibited neointimal thickening (fig. 7c). Digital planimetry revealed a dose-dependent reduction of 42 and 90% in the intima/media ratio, respectively, for each gliotoxin dose (fig. 8a). The decreased intima/media ratio was associated with a reduction of 51 and 88% in the degree of stenosis (fig. 8b). The observed changes depended on the reduction in neointima thickness (fig. 8c), without changes in media thickness (fig. 8d).

Gliotoxin Inhibits the Expression of PCNA in Injured Carotid Artery

PCNA expression was used to assess the effect of gliotoxin on vessel wall cell proliferation. No PCNA-positive cells were observed in the media of normal uninjured and untreated carotid arteries (fig. 9a). PCNA was highly expressed in the neointima and media of arteries from untreated rats 10 days after injury (fig. 9b). Gliotoxin dose-

dependently reduced the number of PCNA-positive cells in the neointima (by 31 and 72% for each dose, respectively) and media (by 55 and 77% for each dose, respectively; fig. 9c–e).

Discussion

Cellular responses to vascular injury, e.g. activation, adhesion, migration and proliferation, are involved in vascular proliferative disorders, e.g. atherosclerosis and intimal hyperplasia after angioplasty, reducing the vessel lumen and leading to restenosis [22–25]. Restenosis following percutaneous transluminal angioplasty has emerged as a complex multifactorial pathophysiological problem that has to be addressed by a combined mechanical and pharmacological approach [3, 25–27]. Animal models showing decreased neointimal thickening [4, 5] provided the basis for controlled clinical trials with sirolimus- or paclitaxel-eluting stents [6, 7, 41–43]. However, the reduction in restenosis events achieved by such stents

Fig. 7. Gliotoxin inhibits neointimal hyperplasia after rat carotid balloon injury. The left common carotid artery was denuded of the endothelium by balloon angioplasty and rats were treated with gliotoxin daily for 10 days. Representative histological cross-

sections of uninjured normal (a), injured untreated (b) or injured carotid artery from a rat treated with 400 μ g gliotoxin/kg body weight/day (c). HE. Original magnification, $\times 100$.

Fig. 8. Gliotoxin significantly reduces the intima/media (I/M) index and the degree of stenosis. Carotid arteries were studied by computer image analysis 10 days after injury. **a** Ratio of I/M cross-sectional areas. **b** Percentage of luminal stenosis of untreated injured (control) rats, and rats treated with 200 (G200) or 400 μ g gliotoxin/kg body weight/day (G400). * $p < 0.005$; ** $p < 0.0001$,

vs. the control group. The decreased I/M index was due to decreased neointimal formation (c), without changes in media thickness (d). * $p < 0.0001$ vs. control. Morphometry was performed from at least five individual sections of arterial segments of each rat. Values are means $\pm$ SD from 8 animals. MU = Metric units squared.

Fig. 9. Gliotoxin inhibits the expression of PCNA in neointima and media of injured carotid arteries. PCNA was assessed on cross-sections from uninjured normal (a), injured untreated (b), or injured carotid artery treated with 200 (G200; c) or 400 (G400) µg gliotoxin/kg body weight/day (d). Representative photomicrographs of carotid sections stained with anti-PCNA antibody. Original magnification, $\times 200$. **e** PCNA-positive cells in neointima and media of injured arteries are expressed as the percentage of total cells. Data are means $\pm$ SD of 8 rats. The value for each rat is the result of studying 8–10 high-power fields in four sections of each artery (three fields in the 400 µg/kg group because of the small amount of thickened intima). * $p < 0.04$, ** $p < 0.0005$, *** $p < 0.001$, **** $p < 0.0003$, vs. injured untreated (control) carotid.

is associated with a higher incidence of late stent thrombosis [8–13]. In this regard, alternative agents providing decreased neointimal thickening should be sought for.

Gliotoxin shows several benefits, including anti-inflammatory and -proliferative activity that may be useful in preventing neointimal formation [15, 17, 21, 44–46]. We explored the actions of gliotoxin on VSMC processes known to contribute to neointima formation. PDGF is a specific mediator of migration of cultured VSMC [30, 31, 47]. Gliotoxin exhibits a powerful antimigratory effect on VSMC, which may be related to its ability to disrupt cytoskeletal proteins. The cytoskeleton mediates essential biological functions, providing a structural framework

and dynamic properties, and it is involved in several pathophysiological processes, including atherosclerosis [48, 49]. Changes in the F-actin cytoskeleton are essential for cell chemotaxis and NF- κ B activation [34, 35, 50]. Gliotoxin disorganized F-actin stress fibers and rearranged the distribution of vimentin, a main component of intermediate filaments. The tail domain of vimentin interacts with actin-containing structures, suggesting a cross talk between both cytoskeletal networks [51]. The effect seems to be specific, because gliotoxin did not modify the distribution pattern of β -tubulin. This observation is consistent with cytoskeletal modulation by gliotoxin in neutrophils [35].

The NF- κ B/Rel family of transcription factors plays an important role in the inducible regulation of a variety of genes involved in inflammatory, proliferative and migratory responses [48, 52]. NF- κ B is commonly expressed in the cytoplasm as an inactive dimer complex, generally formed by the p50 and p65 (RelA) subunits, associated to inhibitory I κ B proteins. The activation of NF- κ B requires degradation of I κ B and nuclear translocation of the cytoplasmic subunits [38, 40, 53]. NF- κ B is activated in proliferating cultured VSMC [39]. Gliotoxin is a powerful and specific inhibitor of the activation of NF- κ B in various cell types [17, 19, 20], but it had not been studied in vascular cells. We here show that gliotoxin blocks the nuclear translocation of the p65 subunit in rat VSMC as well as in endothelial cells, indicating inhibition of NF- κ B activation. NF- κ B is greatly induced in the rat carotid injury model [39], and p65 antisense oligonucleotides reduce neointimal formation [54]. NF- κ B activation has also been found within human atherosclerotic lesions or after angioplasty, but not in normal arteries [38, 55, 56].

The observation that gliotoxin inhibits migration, proliferation and nuclear translocation of the NF- κ B p65 subunit in cultured VSMC might be relevant to the beneficial effect observed in the model of carotid artery damage by angioplasty. Gliotoxin markedly suppresses neointimal development, reducing up to 90% of both the intima/media ratio and the degree of luminal stenosis. Indeed, a potent in vivo antiproliferative action of gliotoxin was documented in the neointima and media of injured vessels. These data provide evidence that gliotoxin confers protection against aberrant VSMC proliferation under pathological states. Gliotoxin decreased VSMC PCNA expression in culture and in vivo. PCNA is both a marker of cell division and a therapeutic target [36, 37] as it is highly expressed in rat carotid artery after balloon injury, and PCNA antisense oligonucleotides limit intimal hyperplasia [18, 57].

Inflammation has been associated with impaired endothelialization or higher restenosis rates after the use of paclitaxel or 32 P-containing stents [58, 59]. Gliotoxin also has direct actions on the endothelium, reducing the expression of NF- κ B-dependent adhesion molecules and leukocyte adhesion in activated cultured endothelial cells. Adhesion molecules are required for adhesion and recruitment of leukocytes at the sites of injury. Indeed, reduced leukocyte infiltration had previously been described in gliotoxin-treated animals with colitis or glomerulonephritis [21, 60]. Indeed, gliotoxin has been shown to decrease inflammation in experimental colitis [61, 62], cornea [63] skin [15] and kidney [21]. In this re-

gard, there was no clear evidence of toxicity in our model or other models [21]. However, gliotoxin is a mycotic toxin and its long-term systemic use in humans might be associated with toxic effects. Its potential toxicity would be decreased by its local release from a drug-eluting stent. The concentrations found to have beneficial effects on VSMC and endothelium (100 ng/ml) are well below those toxic for cells in culture [64] and are within the range found in the serum of patients with invasive aspergillosis [65].

In summary, gliotoxin prevented neointimal formation following balloon injury to carotid arteries. This beneficial effect may be related to direct effects on VSMC, since gliotoxin inhibited VSMC migration, proliferation and NF- κ B activation, as well as on the endothelium, where it decreased leukocyte adhesion. Thus, we have identified new cell targets for gliotoxin actions and a new compound which may be useful to prevent restenosis.

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