Beneficial Effects of NO-Releasing Derivative of Flurbiprofen (HCT-1026) in Rat Model of Vascular Injury and Restenosis

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Abstract—One of the major problems related to the percutaneous transluminal coronary angioplasty technique is the renarrowing of the vessel, a phenomenon known as restenosis. NO and nonsteroidal anti-inflammatory drugs have been shown to play a role in this pathology. The main problem with the use of conventional NO donors is that they affect blood pressure and flow, and for these reasons, they cannot be used safely in clinical practice. The aim of this study was to evaluate, with the use of a rat model of balloon angioplasty, whether a structural derivative of flurbiprofen, containing an added NO-releasing moiety (HCT-1026), is able to reduce or prevent neointimal formation. Rats were treated for 14 days with equimolar doses of flurbiprofen (2, 7, and 21 mg/kg) or HCT-1026 (3, 10, and 30 mg/kg). After this 14-day treatment, HCT-1026 but not flurbiprofen significantly modified the neointima/media ratio. The reduction in the neointimal proliferation obtained with HCT-1026 was well correlated with an increase in nitrite/nitrate plasma levels and a reduced cell proliferation. Neither HCT-1026 nor flurbiprofen affected inducible NO synthase induction in injured vessels. In conclusion, HCT-1026 caused a significant reduction in restenosis that appears to be directly related to NO release. HCT-1026 may prove to be beneficial in preventing or delaying restenosis in humans. (Arterioscler Thromb Vasc Biol. 2002;22:263-267.)

Key Words: NSAIDs ■ HCT-1026 ■ nitric oxide ■ restenosis ■ balloon angioplasty

The present hypothesis involving the pathogenesis of atherosclerotic plaque considers this lesion to be an inflammatory fibroproliferative response of endothelium and smooth muscle of the artery wall to various forms of insult, such as oxidized LDL, bacterial and viral products, or mitogens.1,2 The progression of the atherosclerotic lesion is a consequence of multiple interactions among various cell types (endothelial cells, smooth muscle cells, monocyte/macrophages, T lymphocytes, and platelets) and a large number of vasoregulatory molecules, cytokines, growth factors, and adhesion molecules generated by these cells.1,2 The process involves the migration and proliferation of smooth muscle cells, which elaborate connective tissue within the intima of the affected artery and produce the obliterating atherosclerotic lesion. A mediator that has recently been proposed to be a key modulator of the atherosclerotic process(es) is NO. Indeed, much evidence on the antiatherogenic effect in vivo and in vitro of NO has been accumulated. It has been shown to interfere with several processes involved in atherogenesis. In particular, NO has been shown to inhibit in vitro cell-mediated LDL oxidation,3 smooth muscle cell proliferation,4,5 platelet activation,6 and macrophage apoptosis.7,8 In vivo NO has been shown to modulate endothelial-leukocyte interactions,9 and NO donors have been shown to reduce intimal thickening.10 Percutaneous transluminal coronary angioplasty (PTCA) is a widely used technique in the therapy of coronary artery stenosis in which the occluded segment is dilated by inflating a balloon. One of the major problems related to this technique is the renarrowing of the vessel, a phenomenon known as restenosis. Ideally, delivering NO should reduce the incidence of restenosis or considerably slow down the restenotic process. The main problem with this approach is that conventional NO donors affect blood pressure and flow, and for these reasons, they do not represent a safe pharmacological tool. Recently, a new class of nonsteroidal anti-inflammatory drugs (NSAIDs) with a reduced gastrointestinal toxicity has been described; these are called NO-NSAIDs.11,12 In the present study, by using a new derivative of flurbiprofen, on whose structure has been inserted an NO-releasing moiety (HCT-1026), we have investigated whether NO delivery, through HCT-1026, would prove to be beneficial in an animal model of restenosis.

Methods

Animals

Male Wistar rats (Harlan Italy, San Pietro al Natisone, Udine, Italy) weighing 300 to 350 g were used for the present study. Animals were...
housed in propylene cages with food and water ad libitum. The light cycle was automatically controlled (on at 7:00 AM and off at 7:00 PM), and the room temperature was thermostatically controlled to 22±1°C. Before the experiment, the animals were housed in these conditions for 4 or 5 days to become acclimatized.

Materials

DL-Dithiothreitol, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, pepstatin A, leupeptin, and benzamidine were from Calbiochem. Nonfat dry milk was from Bio-Rad. Anti-inducible NO synthase (anti-iNOS) antibody and anti-mouse immunoglobulins coupled to peroxidase were from Transduction Laboratories. Mouse monoclonal antibodies PC10 and anti-mouse IgG were from Dako. Flurbiprofen nitroxybutyl ester (HCT-1026) was from NicOx SA. All the other reagents were from Sigma Chemical Co.

Endothelial Denudation (Balloon Angioplasty)

Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Angioplasty of the right carotid artery was performed by using a balloon embolectomy catheter (2F; Fogarty; SEDA) according to the procedure described by Indolfi et al. The balloon catheter was introduced through the right external carotid artery into the aortic arch and inflated at 2 atm for 14 days starting the day of the angioplasty. Control animals received an equal volume of PEG (0.2 mL per rat). Rats were housed for 30 minutes in a warmed room (28°C to 30°C); then a tail cuff, consistently ~2 cm from the base of the tail, was placed; and arterial blood pressure was measured immediately after balloon angioplasty (day 0) and on days 7 and 14. Heart rate was detected by a pulse-rate counter placed after the tail cuff.

NOx Assay

Blood specimens were collected, before euthanasia, from all rats by cardiac puncture. Blood was kept at 37°C for 12 hours and serum-separated by using a plastic Pasteur pipette. Samples were kept frozen at ~80°C until they were used. The amount of nitrite/nitrate (NOx) present in serum specimens was determined according to Thomsen et al. After reducing NO3 to NO2 by using acid-washed cadmium powder, NO2 was measured by using a microplate assay method based on the Griess reaction.

Preparation of Total Extracts

The carotid segments were frozen in liquid nitrogen, immediately suspended in 600 μL high salt extraction buffer (20 mMol/L HEPES pH 7.9, 420 mMol/L NaCl, 1.5 mMol/L MgCl2, 0.2 mMol/L EDTA, 25% [vol/vol] glycerol, 1% NP-40, 0.5 mMol/L phenylmethylsulfonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mMol/L benzamidine, and 0.5 mMol/L dithiothreitol), homogenized at the highest setting for 1 minute in a Polytron DT 3000 (Kinematica AG) tissue homogenizer, and incubated on ice for 15 minutes. The homogenates were then centrifuged for 15 minutes at 13,000g, and the supernatant was divided into aliquots and stored at ~80°C. Protein concentration was determined by the Bio-Rad protein assay kit.

Western Blot Analysis

Western blot analysis for inducible NO synthase (iNOS) was performed on total extracts. Equal amounts of proteins (75 μg for each sample) were mixed with gel loading buffer (20 mMol/L Tris, pH 6.8, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, and 2 mg/mL bromophenol) at a ratio of 1:1, boiled for 3 minutes, centrifuged at 10,000g for 10 minutes, and electrophoresed in a 10% discontinuous polyacrylamide minigel. The proteins were transferred to nitrocellulose membranes according to the manufacturer’s instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% nonfat dry milk in PBS and then incubated with anti-iNOS (1:10,000) mouse antibody for 2 hours at room temperature. The membranes were washed 3 times with 1% Triton X-100 in PBS and then incubated with anti-mouse immunoglobulins coupled to peroxidase (1:2000). The immunocomplexes were visualized by the enhanced chemiluminescence method (ECL, Amersham). Subsequently, the relative bands were quantified by densitometric scanning of the x-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Statistical Analysis

Data are expressed as mean±SEM of (n) rats. Statistical significance was calculated by 1-way ANOVA, and a Bonferroni-corrected P value was used for multiple comparisons. The level of statistically significant difference was defined as P<0.05.

Results

Effect of Nitro-Flurbiprofen and Flurbiprofen on Neointimal Proliferation

In sham-operated rats (n=5) not subjected to vascular injury, there was no neointimal formation (Figure 1). In rats sub-

Measurement of Arterial Blood Pressure Indirectly in Conscious Rats

Mean arterial blood pressure in conscious rats was measured by a blood pressure recorder (Ugo Basile, Biological Research Apparatus) by using a technique previously described. After a 1-week training period, the rats were subjected to vascular injury as described above. HCT-1026 (30 mg/kg), freshly dissolved in PEG, was administered orally, once a day for 14 days, starting the day of the angioplasty; the control animals received an equal volume of PEG (0.2 mL per rat). Rats were housed for 30 minutes in a warmed room (28°C to 30°C); then a tail cuff, consistently ~2 cm from the base of the tail, was placed; and arterial blood pressure was measured immediately after balloon angioplasty (day 0) and on days 7 and 14. Heart rate was detected by a pulse-rate counter placed after the tail cuff.

PCNA Analysis

Proliferating cell nuclear antigen (PCNA) analysis was used to quantify the proliferative activity of cells at the balloon injury sites, and it was performed according to Siitonen et al. PCNA-positive cells were counted in the vessel cross sections by using a standard light microscope equipped with an ocular reticle (magnification ×10) and a ×40 objective. At least 500 nuclei were counted from each slide. The sections were photographed under low power, video-digitized, and stored in the image analysis system (Qwin Lite 2.2, Leica). Staining results were expressed as percentage of PCNA-immunoreactive cell nuclei. Faint diffuse nuclear staining seen in some tissue sections was not included in the PCNA score.

Gastric Tolerability

The stomachs of all the rats used in these experiments were removed and examined for hemorrhagic lesions, and a gastric damage score was calculated as previously described. The method involves measurement of the lengths of each lesion in millimeters and summing the lengths of all lesions in each stomach.
Jected to angioplasty and receiving the vehicle, the neointimal area was $0.211 \pm 0.008$ mm$^2$ ($n=16$). HCT-1026 at a dose of 3 mg/kg did not modify neointimal formation, whereas at doses of 10 and 30 mg/kg, it caused a dose-dependent inhibition of neointimal formation by 38% ($n=12$, $P<0.001$) and 45% ($n=5$, $P<0.001$), respectively. On the other hand, flurbiprofen administered at equimolar doses (2, 7, and 21 mg/kg) did not inhibit neointimal formation.

**Quantification of Proliferating Cells by Immunohistochemical Staining**

PCNA is a 36-kDa acidic nuclear polypeptide that is involved in DNA synthesis as a cofactor for DNA polymerase delta. PCNA plays a critical role in the initiation of cell proliferation, and its expression is elevated almost exclusively during the S phase of the cell cycle.14 PCNA-positive cells were not observed in the sections of carotid arteries obtained from sham-operated rats. Furthermore, in all cross sections of injured carotid arteries analyzed, the percentage of PCNA-positive cells in the media was <1%. Thus, only the percentages of positively stained cells in the neointima were used to compare the proliferative activity among groups. In the control group, 14 days after balloon angioplasty, PCNA-positive cells in the neointimal area were $9.0 \pm 0.5\%$ ($n=5$) of the nuclei counted. HCT-1026, at a dose of 30 mg/kg, caused a significant reduction of the percentages of PCNA-positive cells in the neointima ($5.2 \pm 0.6\%$, $n=5$; $P<0.05$). Compared with no treatment, treatment of the rats with flurbiprofen at an equimolar dose (21 mg/kg) did not modify the percentages of PCNA-positive cells ($8.2 \pm 0.7\%$, $n=5$; $P=NS$; Figure 2).

**Gastric Tolerability**

Flurbiprofen elicited dose-dependent gastric damage. Oral administration of flurbiprofen at 2, 7, and 21 mg/kg caused dose-dependent stomach damage; scores were $15.2 \pm 2.0$, $28.1 \pm 4.5$, and $41.2 \pm 8.2$ mm, respectively. Conversely, damage scores after the administration of HCT-1026 were significantly lower ($P<0.01$ versus the correspondent equimolar dose). Doses of 3, 10, and 30 mg/kg HCT-1026 caused gastric scores of $1.2 \pm 0.2$, $2.0 \pm 0.2$, and $8.0 \pm 1.0$ mm, respectively.

**Arterial Blood Pressure**

In control animals ($n=5$), the mean arterial blood pressures on days 0, 7, and 14 were $156.0 \pm 3.4$, $150.5 \pm 4.9$, and $152.2 \pm 2.1$ mm Hg, respectively. No significant changes in blood pressure were observed in animals treated with HCT-1026 (30 mg/kg, $n=5$). The mean arterial blood pressures were $151.9 \pm 2.8$, $154.4 \pm 3.0$, and $157.2 \pm 3.4$ mm Hg on days 0, 7, and 14, respectively.

**NOx Serum Concentration**

The amount of NOx present in serum specimens of sham-operated rats was $12.7 \pm 0.8$ μmol/L ($n=5$), whereas in the control group, the NOx level was $14.3 \pm 1.32$ μmol/L ($n=20$). HCT-1026 at 3, 10, and 30 mg/kg caused a dose-dependent increase of NOx amounts by 14% ($n=5$), 67% ($n=12$, $P<0.001$), and 102% ($n=8$, $P<0.001$), respectively. In contrast, treatments of rats with flurbiprofen (2 to 21 mg/kg, $n=5$ to 8) did not modify the NOx serum concentration.
been confirmed in human vascular tissues, in which the gene
plasty and that mal hyperplasia of the rat carotid artery after balloon angio-
metric analysis (B) showing the effect of HCT-1026 (30 mg/kg)
in injured rat carotid arteries. Results are expressed as mean±SEM of 3 separate experiments.

Effect of Nitro-Flurbiprofen on iNOS
Protein Expression
Balloon injury caused a significant increase in the expression of iNOS in carotid arteries of control rats compared with sham-operated rats (Figure 3). Densitometric analysis showed that HCT-1026 (30 mg/kg) did not reduce iNOS band intensity. Treatment of the rats with flurbiprofen at an equimolar dose (21 mg/kg) did not modify iNOS band intensity.

Discussion
PTCA is a widely used technique in the therapy of coronary artery stenosis. PTCA involves a balloon vasodilatation to restore vessel patency and to improve blood flow. The major problem associated with this technique is restenosis, which affects ≈20% to 50% of the patients treated. One of the major challenges at the present stage of pharmacology is to find a means by which to control the restenotic process. After vascular damage, the rat carotid artery develops a neointimal proliferation that causes a clear narrowing of the vessel lumen, and this experimental technique has been widely used to study the pathophysiology of restenosis and to test drugs. 

NO suppresses several restenotic processes. It has been shown that oral L-arginine supplementation suppresses intimal hyperplasia of the rat carotid artery after balloon angioplasty and that NG-nitro-L-arginine methyl ester can revert the L-arginine effect, indicating that the attenuation of the intimal hyperplasia is mediated by NO, presumably through iNOS. Interestingly, in vivo endothelial NO synthase gene transfer to the denuded rat carotid artery and, more precisely, into the media not only restored the calcium-dependent NO production and the relaxation of the denuded artery but also inhibited neointimal formation by 70% 14 days after balloon injury. The key role played by NO has also been confirmed in human vascular tissues, in which the gene transfer of endothelial NO synthase reduced smooth muscle cell proliferation and neointimal formation. Similarly, iNOS gene transfer has been shown to reduce neointimal formation. Treatment of patients with 3-morpholinosydnonimine before balloon angioplasty followed by prolonged oral administration of molsidomine led to an improvement in long-term lumen patency even though the treatment did not affect the clinical outcome of late restenosis. However, it is technically complicated to deliver NO to the vessels without causing hypotension, and dietary supplementation with L-arginine does not appear to be a feasible approach.

NSAIDs have been shown to have a beneficial effect in restenosis. In particular, aspirin is the most widely prescribed agent for reducing the platelet-mediated contributions to atherosclerosis, coronary thrombosis, and restenosis after angioplasty. Aspirin therapy is limited because it blocks only some of the input stimuli, leaving aspirin-independent pathways untouched, and it has been suggested that new agents that regulate platelet-free cytosolic calcium, such as direct NO donors, may be more potent overall than aspirin. In the present study, we have used a combined approach by using an NO-releasing form of flurbiprofen, a very powerful NSAID. Administration of HCT-1026 but not flurbiprofen led to a reduction of neointimal proliferation, suggesting that inhibition of cyclooxygenase alone, which is expressed in damaged vascular tissue, does not account in this model for a beneficial effect of this particular NSAID. This finding is also in agreement with a recent study in which it was shown that sulindac inhibits neointimal formation independently from cyclooxygenase inhibition. However, the doses of flurbiprofen and HCT-1026 used in the present study have been shown to inhibit platelet aggregation and prostaglandin formation in vivo and in vitro.

In conclusion, we have shown that HCT-1026 but not flurbiprofen reduces neointimal hyperplasia in the rat carotid artery. This effect appears to be linked to NO release, which,
in turn, leads to a reduced cell proliferation. In addition to its beneficial inhibitory effect on the restenotic process, the lack of gastric toxicity may warrant a safe use of this drug as a preventive treatment in restenosis. Alternatively, this compound could also be useful in stent technology, in which the coating of stents with a slow-release form of HCT-1026 may help to prevent or delay restenosis.

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