Suppression of Neointimal Thickening and Smooth Muscle Cell Proliferation After Arterial Injury in the Rat by Inhibitors of Na^+-H^+ Exchange

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Replication of vascular smooth muscle cells is a key event in the pathogenesis of restenosis following angioplasty. Little is known about early biochemical events involved in the proliferation of smooth muscle cells following arterial injury. In the present study, the effect of Na^+-H^+ exchange inhibitors on neointima formation after balloon injury of the rat carotid artery was investigated. Neointima formation was quantified 14 days after injury by morphometric measurement of cross-sectional neointimal area and by fluorometric determination of DNA content. The specific Na^+-H^+ exchange inhibitor 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate (Hoe 694) dose-dependently reduced neointimal area and DNA content, the latter finding indicating a true antiproliferative effect. The structurally different Na^+-H^+ exchange blocker 5-(*N*-ethyl-*N*-isopropyl)amiloride hydrochloride had comparable inhibitory effects on neointimal area and DNA content, whereas 5-methylsulfonyl-2-piperidino-benzoyl guanidine hydrochloride, a position isomer of Hoe 694 lacking Na^+-H^+ exchange blocking properties, did not suppress neointima formation. The effect of Na^+-H^+ exchange blockers on neointima formation depended on the duration of drug application. Maximal suppression was achieved only when Hoe 694 was applied throughout the entire experiment for 14 days. This inhibitory effect of Na^+-H^+ exchange blocker application for the first 2 weeks following injury lasted for 2 months. In conclusion, the results of the present study reveal a potential role of Na^+-H^+ exchange for smooth muscle cell proliferation in vascular disease. (*Circulation Research* 1993;73:264-268)

KEY WORDS • **smooth muscle cells** • **Na^+-H^+ exchange** • **arterial injury** • **arteriosclerosis** • **restenosis**

Proliferation of vascular smooth muscle cells is considered to be the major mechanism underlying the pathophysiology of restenosis after angioplasty.[1] Smooth muscle cell proliferation is thought to be initiated by growth factors released by platelets and by cells of the vessel wall.[2] A great variety of growth factors has been identified that influence smooth muscle cell replication both in vitro and in vivo.[3] Despite their heterogeneity, many of these factors engender a rather uniform response of early biochemical events in the target cell.[4] One of these early changes to mitogenic stimuli is activation of Na^+-H^+ exchange, a cation transport system present in the membrane of almost every mammalian cell type. Activation of Na^+-H^+ exchange results in rapid and sustained cytoplasmic alkalinization.[4-6] Na^+-H^+ exchange activation and subsequent cytoplasmic alkalinization seem to be linked to cell growth and have at least in some cell types a permissive role for mitosis.[5,7] Recently, pharmacological blockade of Na^+-H^+ exchange was found to inhibit DNA synthesis and proliferation of rat aortic smooth muscle cells in culture.[8] So far, the role of Na^+-H^+ exchange for the proliferative response of smooth muscle cells in vivo following arterial wall injury is unknown. The aim of the present study was therefore to investigate the effect of the specific Na^+-H^+ exchange inhibitors 5-(*N*-ethyl-*N*-isopropyl)amiloride hydrochloride (EIPA) and 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate (Hoe 694)[9,10] on neointima formation in the rat carotid artery balloon-injury model.[11]

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Materials and Methods

Animal Model

Male Sprague-Dawley rats weighing 280 to 330 g were obtained from Ivanovas, Kislegg, Germany. Drugs were administered by intraperitoneal injection three times daily starting the day before surgery and continuing until the animals were killed, unless stated otherwise. The drugs were dissolved in 0.9% NaCl, and the solutions were filtered through a 0.2-μm filter (Sartorius, Göttingen, Germany). Control animals received injections with an equal volume of 0.9% NaCl.

Balloon injury of the vessel wall was performed under general anesthesia with ketamine (100 mg/kg body wt

IM, Parke-Davis, Freiburg, Germany) and xylazine (15 mg/kg body wt IM, Bayer, Leverkusen, Germany). The left common carotid artery was deendothelialized by passage of a 2F Fogarty embolectomy catheter (Baxter, Santa Ana, Calif) as described earlier.[11]

The animals were killed 14 days or, in one experiment, 2 months after the ballooning procedure. The right and the left carotid artery were excised and dissected free of adventitial tissue. The common carotid arteries were divided into three segments of 5-mm length each. The proximal and distal parts were stored in sterile DNA buffer ([mol/L] NaCl, 2; NaH_2PO_4, 0.05; and EDTA, 0.001; pH 7.4) for DNA quantification. The middle segment of the vessel was fixed by immersion in 4% paraformaldehyde for 4 hours and then stored in phosphate-buffered saline (pH 7.4) for morphometry.

Morphometry

Vessels were embedded in methacrylate (Technovit 7100, Kulzer, Friedrichsdorf, Germany), cross-sectioned, and stained with toluidine blue. The cross-sectional area of the neointima was quantified using a computer-based morphometry system.[12]

DNA Quantification

DNA content per 5-mm vessel wall was measured by the method of Labarca and Paigen.[13] The tissue was homogenized in 2 mL DNA buffer, and aliquots were incubated with the DNA-binding dye Hoechst 33258 (dissolved in DNA buffer; final concentration, 160 ng/mL). Fluorescence (peak excitation wavelength, 365 nm; peak emission wavelength, 460 nm) was measured with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif) using herring sperm DNA (Boehringer, Mannheim, Germany) as standard. Net increase in DNA in the injured left carotid artery (ΔDNA) was calculated with the following formula:

$$\Delta DNA = DNA_{\text{left carotid}} - DNA_{\text{right carotid}}$$

This formula is based on equal amounts of DNA in the media of both uninjured right and left carotid arteries and on the assumption of a constant amount of DNA per intimal cell. Although it does not reflect the DNA synthesis necessary to replace medial cell loss, the equation allows an estimation of inhibitory effects on overall smooth muscle cell proliferation after ballooning. This is possible because in this model of arterial injury the increase in DNA in the whole vessel wall is almost entirely due to cell replication and only to a very minor degree to cell polyploidy,[14] and the majority of neointimal cells are smooth muscle cells.[15]

Reagents

Hoe 694, its position isomer 5-methylsulfonyl-2-piperidino-benzoyl guanidine hydrochloride, and EIPA were gifts of Hoechst, Frankfurt, Germany. Hoechst 33258 was purchased from Sigma, Deisenhofen, Germany.

Statistical Methods

Results were calculated as arithmetic mean±SEM. Statistical difference was examined by analysis of variance followed by Fisher's test.

Results

The Na^+-H^+ exchange inhibitor Hoe 694 significantly suppressed neointima formation 14 days after balloon injury of the rat carotid artery. Administration of the drug starting the day before ballooning until the end of the experiment significantly reduced cross-sectional area of the neointima (3×5 mg/kg body wt, −24.5%; 3×15 mg/kg body wt, −50.5%; and 3×30 mg/kg body wt, −61.7%; Figs 1 and 2). This reduction in neointimal size was paralleled by a similar inhibition of injury-induced DNA increase (ΔDNA) in the left carotid artery (−18.1%, −48.1%, and −57.7%, respectively; Fig 1, Table). Comparable effects on neointima formation were likewise seen with 3×5 mg/kg body wt EIPA (area, −22.9%; ΔDNA, −24.0%; Fig 1, Table). On the other hand, the Hoe 694 isomer 2-methylsulfonyl-5-piperidino-benzoyl guanidine hydrochloride (3×15 mg/kg body wt), which does not inhibit Na^+-H^+ exchange, had no effect on either neointimal size (0.151±0.007 mm^2, n=8; control, 0.159±0.013 mm^2, n=8; P=NS) or DNA increase (3.90±0.17 μg/5 mm; control, 4.13±0.24 μg/5 mm; P=NS). The DNA content of the right carotid arteries was not affected by the drugs (Table), suggesting that the inhibitory effect in the injured artery was not due to mere cytotoxicity to smooth muscle cells. In addition, with the doses used for Hoe 694 and EIPA, the animals showed no signs of major general toxicity, as assessed by weight gain over 2 weeks and blood count (data not shown).

The effect of Na^+-H^+ exchange inhibition on neointima formation at 14 days after injury depended on the duration of drug application. Administration of Hoe 694 (3×15 mg/kg body wt) starting the day before ballooning until day 4 after injury resulted in a reduction in neointimal area of −20.9% and in DNA accumulation of −26.0% as compared with the respective control conditions. If drug application was extended to day 7 after injury, there was an increase in the inhibitory effect to −39.5% (neointimal area) and −35.3% (ΔDNA). Maximal inhibition (see above) was achieved if application of Hoe 694 continued throughout the entire experiment (Fig 3). The effect on neointima formation of Hoe 694 (3×15 mg/kg body wt) over 14 days after injury was still present 2 months after ballooning (neointimal area, 0.119±0.008 vs 0.188±0.011 mm^2 [P<.05]; ΔDNA, 2.39±0.32 vs 4.11±0.24 μg/5 mm [P<.05]; n=6 for each group).

Discussion

The results of the present study are compatible with a role of Na^+-H^+ exchange for the proliferative response of smooth muscle cells after arterial injury in the rat.

Hoe 694, which has previously been shown to be a potent blocker of Na^+-H^+ exchange,[10] suppressed neointimal thickening 14 days after balloon injury in a dose-dependent manner. A position isomer of Hoe 694 lacking Na^+-H^+ exchange-blocking properties had no effect on neointima formation. Moreover, EIPA, another specific Na^+-H^+ exchange inhibitor,[16] which is structurally different from Hoe 694, exerted a similar inhibitory effect on neointimal thickening. Thus, it seems unlikely that ancillary properties of Hoe 694, which has been applied in high doses in this study, are responsible for the observed effect, and reduced neoin-

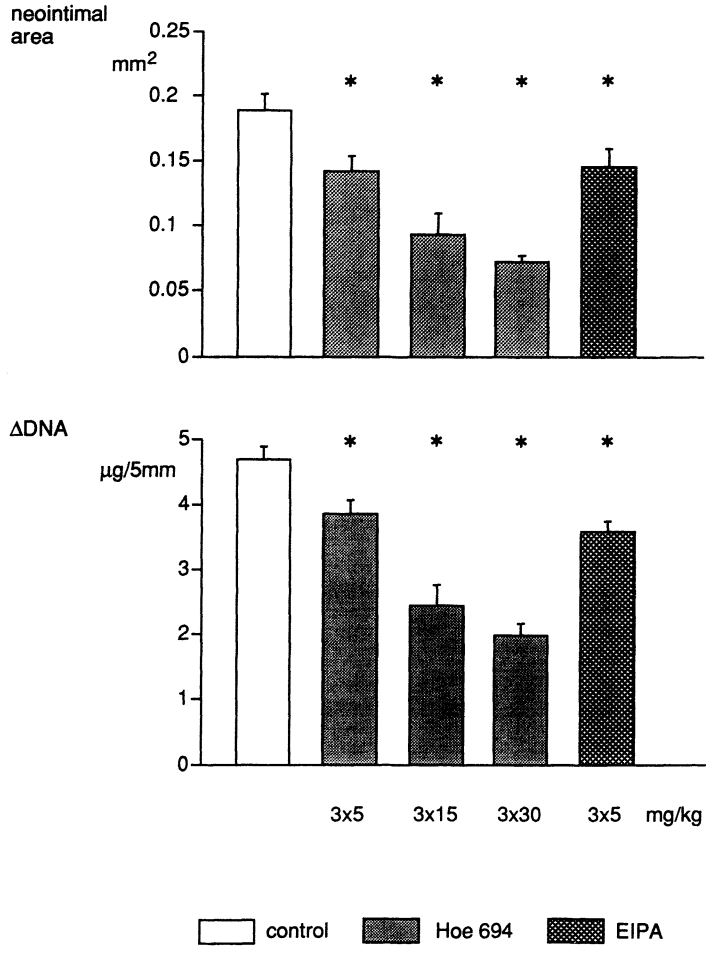

FIG 1. *Bar graphs showing the effect of Na^+-H^+ exchange inhibitors on neointima formation 2 weeks after balloon injury of the left common carotid artery of the rat. Animals (n=6 to 8) received various doses of 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate (Hoe 694, 3×5, 3×15, and 3×30 mg/kg body wt per day) or 5-(N-ethyl-N-isopropyl)amiloride hydrochloride (EIPA, 3×5 mg/kg body wt per day) by intraperitoneal injection starting the day before ballooning and continuing throughout the experiment. Control animals (n=18) were injected with 0.9% NaCl. Cross-sectional neointimal area (top) was measured by morphometry. DNA content of the left and right carotid arteries per 5-mm vessel length (bottom) was determined by fluorometry, and the injury-induced net increase in DNA (ΔDNA) was calculated as described in "Materials and Methods." Values are mean+SEM; *P<.05 vs control value.*

timal thickening can be attributed to a specific effect of Na^+-H^+ exchange blockers.

The inhibition of neointima formation by Na^+-H^+ exchange blockers could result from interaction with several different events that take place after arterial injury in the rat in a sequential manner[11,14,17]: First, smooth muscle cells in the media start to divide (first wave). Thereafter, medial smooth muscle cells migrate into the intimal space (second wave), where a subfraction of these cells also proliferates (third wave). Finally, luminal stenosis is further increased by extracellular matrix production of the cells. This study provides clear evidence for an antiproliferative effect of Na^+-H^+ exchange inhibitors. Parallel to the reduction in neointimal area, the increase in DNA content of the left carotid artery was similarly diminished by Hoe 694 and EIPA but not by the inactive isomer of Hoe 694. Since in this model DNA content of the vessel wall reflects total cell number,[14] these findings suggest that blockers of Na^+-H^+ exchange inhibit cell proliferation in the vessel wall.

The vast majority of neointimal cells in the injured rat carotid artery are smooth muscle cells.[15] In vitro studies show a concentration-dependent inhibition of rat aortic smooth muscle cell proliferation and DNA synthesis by EIPA and other amiloride derivatives.[8] Similarly, Hoe 694 inhibited proliferation of human aortic smooth muscle cells in a concentration-dependent manner (E. Pestel, R. Kranzhöfer, E. von Hodenberg, unpublished observations). Together with these findings, the present data suggest a direct inhibition of smooth muscle cell proliferation in vivo by the Na^+-H^+ exchange blockers.

Any possible interactions with cell migration or extracellular matrix production have not been addressed in this investigation. However, sole inhibition of extracellular matrix synthesis as a possible main effect of Na^+-H^+ exchange inhibitors is unlikely for two reasons: (1) Direct blockade of matrix synthesis may result in a decrease in neointimal area but would not affect DNA content. (2) Matrix production has been found to contribute to neointimal thickening predominantly after the second week following injury.[11]

Whether Na^+-H^+ exchange inhibitors in addition to proliferation affect cell migration is a question of interest for future studies, since smooth muscle cell migration substantially contributes to the neointimal cell mass in this model.[17] Also, more detailed kinetic analyses should reveal whether smooth muscle cell proliferation in the media (first wave), the neointima (third wave), or both are suppressed by Na^+-H^+ exchange blockers.

The degree of inhibition of neointima formation depended on the duration of Na^+-H^+ exchange blockade after injury. Maximal reductions in neointimal area and DNA content were observed only when Hoe 694 was applied throughout the whole course of the experiment until 14 days after ballooning. Although medial and intimal smooth muscle cell proliferation are maximal during the first week after injury, proliferation

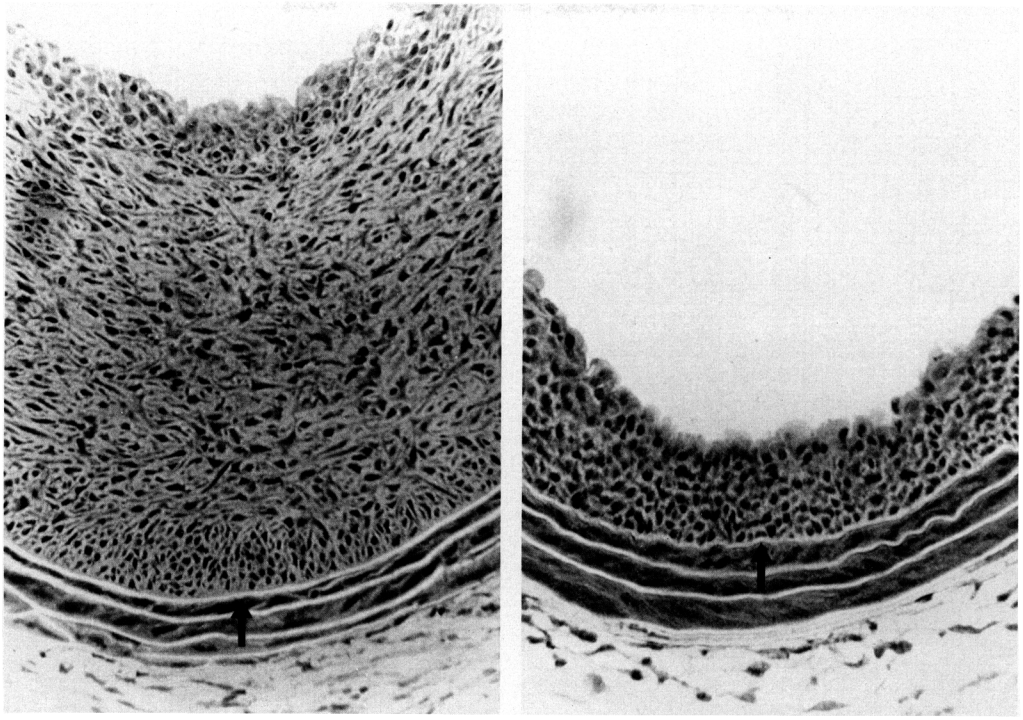

FIG 2. *Photomicrographs showing representative neointimal lesions 14 days after balloon catheter injury of the left common carotid artery of a rat injected with 0.9% NaCl (left) and of a rat receiving 3×30 mg/kg body wt per day of the Na^+-H^+ exchange inhibitor 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate (Hoe 694, right) from the day before injury until the end of the experiment. Arrows indicate the internal elastic lamina between media and intima. Toluidine blue staining, original magnification ×210.*

rates, as estimated by measuring [^{3}H]thymidine incorporation, are still above normal during the second week.[14] This can explain the ongoing effectiveness of Na^+-H^+ exchange blockers during the second week after ballooning, since each new mitotic cycle should be accompanied by temporary activation of Na^+-H^+ exchange. On the other hand, if the Na^+-H^+ exchange blocker was administered for the first 2 weeks after arterial injury and then discontinued, the full inhibitory effect was still present 2 months after ballooning. This suggests that the action of Na^+-H^+ exchange blockers during a sensitive period early after injury definitely prevents cells from undergoing mitosis and does not merely retard entry into the cell cycle.

Smooth muscle cell proliferation, believed to be the major mechanism finally leading to restenosis after angioplasty, probably results from many different growth factors and cytokines acting both sequentially and in concert. Among the many factors identified as contributing to neointima formation during the past few years are several known to activate Na^+-H^+ exchange in vascular smooth muscle cells, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and angiotensin II. Administration of antibodies against PDGF or bFGF or of angiotensin converting enzyme inhibitors or angiotensin II receptor blockers suppressed neointima formation by inhibition of smooth muscle cell proliferation or migration.[18-21] In the present study, agents that interfere with a possible common signaling pathway suppressed neointimal thickening and smooth muscle cell proliferation after arterial injury. There are other pathophysiological mechanisms underlying cardiovascular diseases, such as cardiac hypertrophy and angiogenesis (eg, in diabetic retinopathy), in which growth

Effect of Na^+-H^+ Exchange Inhibitors on DNA Content in the Carotid Artery at 14 Days

		DNA (μg/5-mm vessel)		
Treatment	n	Left carotid	Right carotid	Δ (left−right)
Control (0.9% NaCl)	18	8.32±0.20	3.62±0.14	4.71±0.17
Hoe 694				
3×5 mg/kg	8	7.88±0.22*	4.04±0.12	3.86±0.22*
3×15 mg/kg	7	6.52±0.45*	4.08±0.16	2.44±0.34*
3×30 mg/kg	6	5.81±0.23*	3.82±0.15	1.99±0.17*
EIPA (3×5 mg/kg)	8	7.32±0.11*	3.75±0.13	3.58±0.18*

Hoe 694, 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride hydrochloride. Values are mean±SEM.

*$P<.05$ vs respective control value.

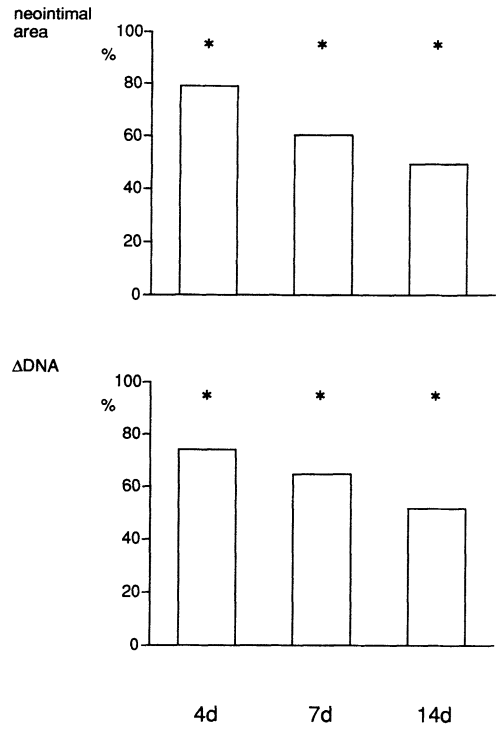

FIG 3. *Bar graphs showing the effect of various intervals of application of the Na^+-H^+ exchange inhibitor 3-methylsulfonyl-4-piperidino-benzoyl guanidine mesylate (Hoe 694) on neointima formation 2 weeks after balloon injury of the left common carotid artery of the rat. Animals (n=5 to 7) received Hoe 694 (3×15 mg/kg body wt per day) by intraperitoneal injection starting the day before ballooning until day 4 or day 7 after the procedure or throughout the experiment. Control animals (n=4 to 18) were injected with 0.9% NaCl. Cross-sectional neointimal area (top) was measured by morphometry. DNA content of the left and right carotid arteries per 5-mm vessel length (bottom) was determined by fluorometry, and the injury-induced net increase in DNA (ΔDNA) was calculated as described in "Materials and Methods." Results are given as percentage of the respective control value.* *P<.05 *vs respective control value.*

factors are thought to play a role and, hence, Na^+-H^+ exchange can be activated. Pharmacological inhibition of a common pathway for those growth factors, such as blockade of Na^+-H^+ exchange, might be a more effective approach than blocking single factors.

The exact mechanism by which Na^+-H^+ exchange activation contributes to cell division is still poorly understood. Recently, inhibition of Na^+-H^+ exchange was demonstrated to abolish late but not early growth factor–stimulated protein synthesis in cultured rat aortic smooth muscle cells.[8] It was therefore concluded that Na^+-H^+ exchange blockade prevents progression of the cell to the S phase late in the G_1 phase of the cell cycle. According to a recent study,[22] Na^+-H^+ exchange activity is required for growth factor–induced gene expression of the M1 and M2 subunits of ribonucleotide reductase, an enzyme critical for DNA synthesis.

In summary, the present data suggest that Na^+-H^+ exchange is involved in proliferative vascular disease. Therefore, Na^+-H^+ exchange may be a target for new antiproliferative strategies.

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