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# Effects of local delivery of trapidil on neointima formation in a rabbit angioplasty model

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**1** Smooth muscle cell (SMC) proliferation can result in luminal reduction of a vessel following balloon angioplasty. This study was designed (i) to determine if local administration of trapidil (triazolopyrimidine) into a vessel wall reduces neointima formation, and (ii) to explore the mechanism involved in the subsequent reduction in cell proliferation.

**2** Following balloon angioplasty in 40 anaesthetized New Zealand White rabbits, trapidil (50–200 mg) or its vehicle (saline) was injected into the dilated vessel wall of the right femoral artery. Experimental groups and time of investigation: (I) vehicle (2 weeks, $n=3$), (II) trapidil-100 mg (2 weeks, $n=3$), (III) vehicle (3 weeks, $n=8$), (IV) trapidil-50 mg (3 weeks, $n=5$); (V) trapidil-100 mg (3 weeks, $n=9$) or (V) trapidil-200 mg (3 weeks, $n=7$).

**3** After 2 weeks, there was a significant reduction of intimal hyperplasia (expressed as intima to media area ratio) in the trapidil group compared with vehicle ($0.44\pm0.04$ vs $0.93\pm0.04$, $^*P<0.05$) and also a significant reduction in cell proliferation (% ratio of BrdU-positive cells to total cell number: vehicle $14\pm2\%$ vs trapidil $6\pm1\%$, $^*P<0.05$).

**4** After 3 weeks, there was a dose-dependent reduction of intimal hyperplasia in the trapidil groups compared with vehicle (trapidil 50 mg $1.14\pm0.04$; trapidil 100 mg $0.91\pm0.09$*; trapidil 200 mg $0.77\pm0.09$* vs vehicle $1.67\pm0.23$, $^*P<0.05$).

**5** Thus, the local administration of trapidil to the rabbit femoral artery reduces the neointima formation, which occurs 2 or 3 weeks after balloon angioplasty *via* a mechanism, which is dependent on inhibition of cell proliferation.

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**Keywords:** Angioplasty; apoptosis; femoral artery; histo-(patho)-logy; infusion balloon catheter; neointima formation; New Zealand White rabbit; smooth muscle cells; trapidil

**Abbreviations:** BrdU, bromo-deoxyuridine; IMR, intima to media area ratio; MAP kinase, mitogen activated protein kinase; NO, nitric oxide; PKA, protein kinase A; PTCA, percutaneous transluminal coronary angioplasty; SMC, smooth muscle cell(s); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling

## Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a well established intervention for the treatment of coronary artery stenosis due to atherosclerosis. Despite a primary success rate of 90–95%, restenosis occurs in 30–60% of patients (Califf *et al*., 1991; McBride *et al*., 1988). Early restenosis (hours to days after PTCA) may occur due to thrombosis or to a lesser degree, due to elastic recoil or vasospasm (Fischell, 1990; Harker, 1987). Late restenosis, which occurs at 2–6 months after angioplasty, is due to smooth muscle cell (SMC) migration and proliferation in response to balloon injury (Austin *et al*., 1985; Dartsch *et al*., 1989; Farb *et al*., 1990; Gravanis & Roubin, 1989; Leimgruber *et al*., 1986). The exact mechanism of arterial renarrowing after angioplasty is still unknown but may result from both intimal hyperplasia and arterial remodeling (Birnbaum *et al*., 1997; Post *et al*., 1994). Studies in different animal models and in human demonstrated that remodelling is a major determinant of arterial vessel lumen size (Birnbaum *et al*., 1997; Post *et al*., 1994).

It has been postulated that SMC proliferation is controlled by the balance between growth factors including thromboxane ($TXA_2$), serotonin, platelet-derived growth factor (PDGF), transforming growth factor (TGF), fibroblast growth factor (FGF) and growth inhibitors such as prostacyclin ($PGI_2$) and nitric oxide (NO) in the circulation and/or at local tissue sites (Schini-Kerth *et al*., 1996; Scott-Burden & Vanhoutte, 1993; 1994; Zucker *et al*., 1995). If this hypothesis is correct, interference with growth factor mediated cellular hyperplasia may be beneficial in inhibiting SMC proliferation and, therefore may be beneficial in the treatment of restenosis.

Trapidil (triazolopyrimidine) has been shown to reduce neointima formation after PTCA in humans (Maresta *et al*., 1994; Okamoto *et al*., 1992) or in atherosclerotic rabbits (Liu *et al*., 1990). It also reduces the cellular proliferation induced by PDGF in SMC culture, and the intimal thickening in carotid arteries from rats after vessel injury (Ohnishi *et al*., 1981; Tiell *et al*., 1983). The mechanism by which trapidil reduces restenosis is, however, not entirely clear. Recently, it was demonstrated that trapidil exerts its antimitogenic effects by activation of protein kinase A (PKA) *in vitro* (Bonisch *et al*., 1998).

Despite inhibition of proliferation, induction of apoptosis by trapidil may serve as another mechanism of action for prevention of neointima formation. NO donors such as SIN-1, Cas-1609 or sodium nitroprusside induce apoptosis in SMC, and SIN-1 has been shown to reduce the restenosis caused by balloon angioplasty in the rabbit (Grosser *et al*., 1998; Ibe *et*

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al., 1997). Thus, it is conceivable that the induction of SMC apoptosis could contribute to the prevention of neointima formation.

The aim of this study was (i) to determine whether local administration of trapidil reduces intimal proliferation and luminal narrowing after balloon injury in the rabbit femoral artery, (ii) to investigate *in vivo* whether the mechanism of reducing neointima formation is secondary to inhibition of proliferation or due to induction of cell apoptosis, and (iii) to explore *in vitro* whether trapidil inhibits proliferation or induces apoptosis in SMC in culture.

## Methods

### *Animals*

Forty New Zealand White male rabbits (2.8–4.0 kg) were studied. All animals were housed individually in a controlled-temperature, standard light/dark environment and allowed to stabilize before any intervention. The rabbits were randomized to six different groups of angioplasty: (I) Vehicle (saline) group (2 weeks of investigation, $n=3$); (II) Trapidil-100 mg treated group (2 weeks of investigation, $n=3$); (III) Vehicle (saline) group (3 weeks of investigation, $n=8$); (IV) Trapidil-50 mg treated group (3 weeks of investigation, $n=5$); (V) Trapidil-100 mg treated group (3 weeks of investigation, $n=9$); and (VI) Trapidil-200 mg treated group (3 weeks of investigation, $n=7$).

In all groups the intima to media area ratio (IMR) was measured and the rate of apoptosis determined as described below. In groups (I) and (II), the proliferation of SMC *in vivo* was measured using the incorporation of bromo-deoxyuridine (BrdU) as described below.

The animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of University of Mainz, and which complied with the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health, NIH publication No. 85-23, revised 1996).

The *n*-numbers in the above experimental groups refer to animals, which survived until the end of the experiment. The number of animals which died in the individual groups of animals were as follows: group (III): $n=2$, group (IV); $n=2$ and group (V): $n=1$. The average mortality was approximately 13%.

### *Instrumentation*

The Transport Coronary Dilatation-Infusion Catheter used in this study was a triple lumen catheter. One lumen was used for inflation of the balloon, one lumen allowed the infusion of solutions, and the third lumen allowed the use of a guide wire. The balloon design consisted of a balloon within a balloon. The inner balloon provided an inflatable segment of diameter 2.5 mm using a pressure of 6 atmospheres (atm), and a length of 2 cm. The outer balloon had approximately 50 holes located circumferentially along the mid-section of the balloon for the controlled, selective infusion of solutions. In this study we used an infusion pressure of 3 atm (for further details, please refer to the instructions for use of the catheter of the supplier).

### *Angioplasty*

Rabbits were pre-anaesthetized with xylazine hydrochloride (4 mg $kg^{-1}$ i.m.). After 20 min, anaesthesia was induced with a bolus injection of sodium pentobarbital (10 mg $kg^{-1}$ i.v.) and maintained with supplementary injections of 2–4 mg $kg^{-1}$ doses as required. All experiments and animal handling were conducted in such a manner as to minimize stress and discomfort to the animals.

Rabbits were positioned in a perspex brace in the supine posture, with the hind legs externally rotated and abducted and the knees in full extension. Under sterile conditions the right femoral artery was isolated and an arched incision of 2 cm length in the knee region was made. A femoral arteriotomy was performed, and a 6 F PTCA Dilatation Infusion Catheter was advanced retrogradely to an area within the femoral artery, ascertained by anatomic landmarks (the middle of the balloon was positioned 3 cm from the artery incision) and controlled using angiography. Thereafter, the inner balloon was inflated with saline to a pressure of 6 atm and the outer balloon was infused with different doses of trapidil (50, 100 or 200 mg in 2 ml saline) or vehicle (saline) alone. After 2 min the balloon catheter and deflated and removed. The femoral artery was repaired and the patency of the vessel was controlled manually distal of the suture. The surgical incision was closed, the animals received fragmin (30 I.U. $kg^{-1}$ s.c.), and acetylsalicylic acid (30 mg $kg^{-1}$ i.v.). In addition, animals were also given gentamicin during (6 mg $kg^{-1}$ i.v.) and after the procedure (6 mg $kg^{-1}$ i.m.). The left femoral artery was used in all animals as a control vessel.

### *Tissue preparation and analysis*

Two or three weeks after angioplasty, each rabbit was anaesthetized as described above and both femoral arteries were isolated. After application of heparin (600 I.U. $kg^{-1}$ i.v.), the animals were killed with an injection of sodium pentobarbital (40 mg $kg^{-1}$ i.v.). Both femoral arteries were excised, as well as thymus, mucosa of duodenum, bone marrow, and testicle. After preparation the tissue samples were immersed in paraformaldehyde (4% w $v^{-1}$, for 1 h) and then in phosphate buffered saline (PBS). After dehydration using increasing concentrations of acetone (50, 70, 90 or 100%), the segments were embedded in methacrylate (Mozdzen & Keren, 1982) and then cut into 3 $\mu$m sections.

### *Determination of intima to media area ratio* in vivo

All vessel sections were stained with haematoxylin and eosin. Two matching sections from the injured portion of each artery were examined in a blinded fashion using computer-assisted morphometry (Optimas 6, Optimas Corporation, Washington, U.S.A.). Measurements were made of the cross-sectional area of the lumen and of the areas enclosed by the internal and external elastic laminae. The intimal cross-sectional area of femoral artery segments was determined by subtracting the area of the lumen from the area enclosed by the internal elastic lamina. The medial area was determined by subtracting the area enclosed by the internal elastic lamina from the area enclosed by the external elastic lamina.

### *Determination of the degree of apoptosis* in vivo

Two different methods were used to determine the degree of apoptosis. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) procedure was used to stain nuclei that contained nicked DNA, a characteristic exhibited by cells in the end stages of apoptotic cell death.

Embedded sections of the femoral arteries were investigated using the TUNEL-method following the instructions supplied with the assay (ApopTag, Oncor, U.S.A.).

The Hoe 33342 staining allows visualization of apoptotic cells and demonstrates morphological characteristics such as cell membrane convolutions, e.g. blebbing, nuclei shrinkage and chromatin condensation as well as formation of apoptotic bodies. Embedded sections of the femoral arteries were washed in PBS and then incubated with Hoe 33342 (bisBenzimide: (2′-[4-ethoxyphenyl] - 5-[4 - methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole) trihydrochloride, 10 $\mu$g ml$^{-1}$ for 7 min at room temperature). The sections were then washed twice in PBS and finally mounted.

The degree of apoptosis using both methods was examined using light (fluorescent) microscopy (magnification: 40 and/or 100). One hundred cells in three separate fields of vision were counted, the number of apoptotic cells determined, and expressed in per cent (Gorman *et al*., 1996).

### *Determination of bromo-deoxyuridine (BrdU)-incorporation* in vivo

Immunochemical detection of BrdU incorporation into DNA is a powerful tool for identifying cells in which DNA synthesis has occurred (Gratzner, 1982; Vanderlaan & Thomas, 1985). Therefore, BrdU (40 mg kg$^{-1}$ i.v.) was administered 24 and 2 h before harvest (2 week group). The embedded tissue sections were then examined using the In Situ Cell Proliferation Kit following the instructions supplied with the assay.

### *Preparation of smooth muscle cells*

Medial explants were dissected from freshly harvested rabbit aortic strips and plated in 100 mm Petri dishes as described (Jarmolych *et al*., 1968). Aortic SMC were grown from explants in Dulbecco's Modified Essential Medium (DMEM) containing 10% bovine calf serum, 100 $\mu$g ml$^{-1}$ penicillin, and 100 $\mu$g ml$^{-1}$ streptomycin. Explant derived cells were initially passaged after pretreatment with trypsin (0.05% w v$^{-1}$) EDTA (0.02% w v$^{-1}$) for 5 min at 37°C. Cells at passage 3–8 were used for all growth studies. The authenticity of the aortic SMC cultures was verified using a mouse monoclonal antibody specific for smooth muscle actin. Cells were subcultured into 96-well culture plates in medium containing serum (10% v v$^{-1}$) for 24 h at an initial density of $5 \times 10^{-4}$ cells per well. The cells were then placed in DMEM containing serum (0.5% v v$^{-1}$) for 48 h to render them quiescent.

### *Determination of SMC proliferation and apoptosis* in vitro

SMC proliferation was determined using the Cell Proliferation ELISA kit. SMC apoptosis was determined using the Cell Death Detection ELISA$^{plus}$ kit. Trapidil (1, 3, 10, 30, 100, 300, 1000, 3000 or 10,000 $\mu$g ml$^{-1}$) or its vehicle were added directly into the culture wells, which were incubated for 24 h at 37°C. The BrdU (apoptosis) measurement was performed by following the instructions of the assays. The absorbance was measured at 450 nm using a scanning multiwell spectrophotometer. The developed colour and thereby the absorbance values directly correlate to the amount of DNA synthesis and amount of apoptosis. However, apoptosis was induced using actinomycin D (250 ng ml$^{-1}$) as a reference.

### *Drugs, materials and assays*

The following drugs, materials and assays were used: Transport Coronary Dilatation-Infusion Catheter (Transport®, Boston Scientific Corporation, U.S.A.), xylazine hydrochloride and acetylsalicylic acid (Bayer AG, Leverkusen, Germany), sodium pentobarbital (Sanofi Winthrop, Muenchen, Germany), fragmin (Grünenthal, Aachen, Germany), gentamicin and heparin (Ratiopharm, Ulm, Germany), methacrylate (Immuno-Bed™ Kit, Polysciences Inc., Warrington, U.S.A.), BrdU, In Situ Cell Proliferation Kit, Cell Proliferation ELISA kit, BrdU and Cell Death Detection ELISA$^{plus}$ kit (Boehringer Mannheim, Mannheim, Germany), trapidil (UCB Pharma, Kerpen-Sindorf, Germany), multiwell spectrophotometer (Bio-Tek Instruments Inc., Winooski, U.S.A.). Unless otherwise stated all other compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

### *Statistical analysis*

All values in the text and figures are expressed as the mean ± s.e.mean of $n$ observations. Statistical analysis was performed (on absolute values) by one-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni's test for multiple comparisons. A $P$ value of less than 0.05 was considered statistically significant.

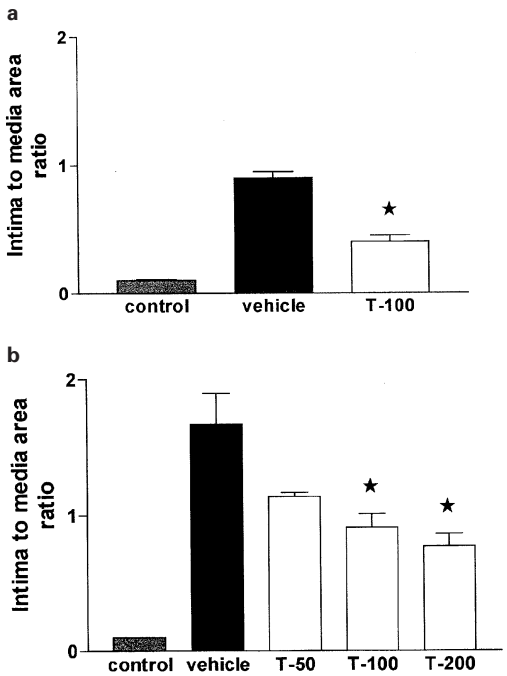


**Figure 1** Neointima formation *in vivo* expressed as intima to media area ratio. Values are measured 2 weeks (Figure 1a) or 3 weeks (Figure 1b) after balloon angioplasty of the rabbit femoral artery. (a) Controls ($n$ = 6, no angioplasty) show a normal ratio for an uninjured vessel. In contrast, angioplasty in the vehicle group ($n$ = 3) leads to an intimal hyperplasia with increasing values for the intima to media area ratio. Trapidil (T-100, 100 mg local injection into the femoral artery, $n$ = 3) reduces intimal hyperplasia significantly compared to vehicle (*$P$ < 0.05 when compared to vehicle). (b) Controls ($n$ = 8, no angioplasty) show a normal ratio for an uninjured vessel. In contrast, angioplasty in the vehicle group ($n$ = 8) leads to an intimal hyperplasia with increasing values for the intima to media area ratio. Local administration of trapidil into the femoral artery caused a dose dependent reduction in intima to media area ratio compared to vehicle (T-50, 50 mg ($n$ = 5); T-100, 100 mg ($n$ = 9); T-200, 200 mg ($n$ = 7)), (*$P$ < 0.05 when compared to vehicle).

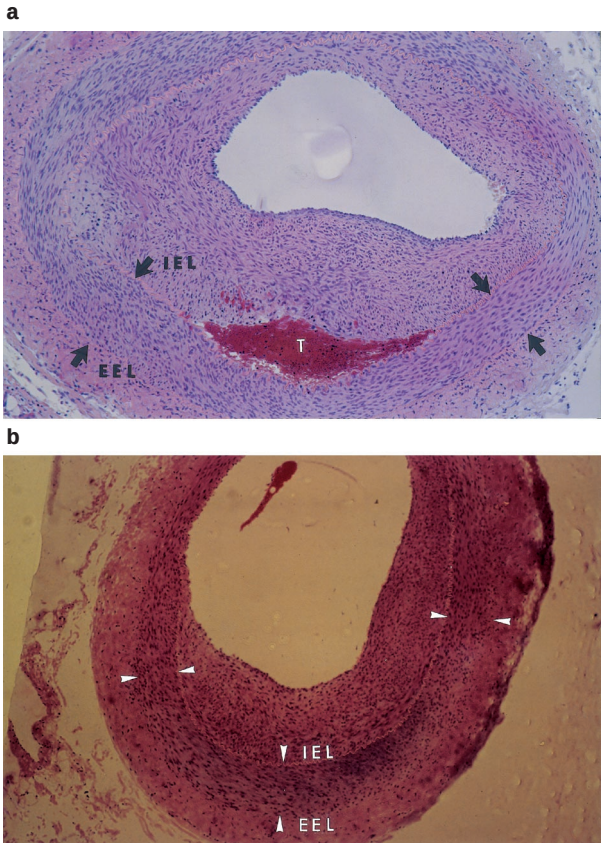


**Figure 2** Haematoxylin-eosin staining of a typical histological section of the right femoral artery of a rabbit. (a) and (b) depict a vessel 3 weeks after balloon injury, external elastic lamina (EEL) and internal elastic lamina (IEL) are marked with arrows, the lumen of the vessels are not concentric. There is an extensive neointima within the lamina elastica interna. In addition, a formation of a thrombus (T) is visible within the neointima (a). (b) Shows a typical example of vessel 3 weeks after balloon injury of a trapidil treated animal. The neointima is well visible within the internal elastic lamina but less extended when compared to a control vessel (a).

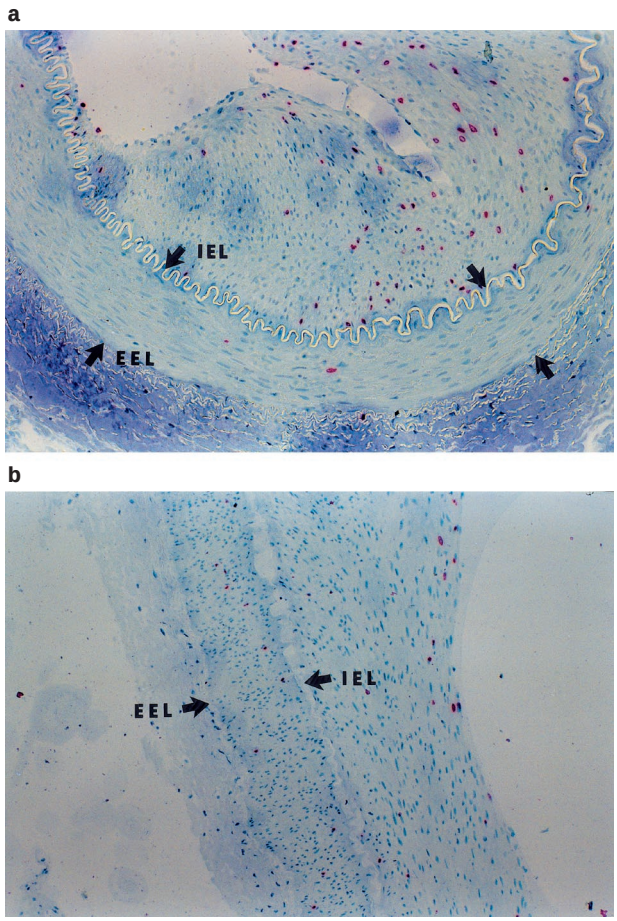


**Figure 3** Bromodeoxy-uridine (BrdU) staining of a typical histological section of the right femoral artery of a rabbit. (a) and (b) depict a vessel 2 weeks after balloon injury, external elastic lamina (EEL) and internal elastic lamina (IEL) are marked with arrows. There is an extensive neointima formation within the lamina elastica interna (a). Additionally proliferative cells are stained red. (b) shows a typical example of vessel 2 weeks after balloon injury of a trapidil treated animal. It should be noted that within the neointima there is less proliferation visible, depicted as a reduction of the number of nuclei containing red stain.

**Table 1** Cross-sectional area measurements if intima and media of rabbit arterial vessels subjected to angioplasty

| | *Intima area* ($mm^2$) | *Media area* ($mm^2$) |
|---|---|---|
| Vehicle ($n=8$) | $0.80\pm0.11$ | $0.48\pm0.05$ |
| Trapidil 50 mg ($n=5$) | $0.62\pm0.09$ | $0.54\pm0.01$ |
| Trapidil 100 mg ($n=9$) | $0.32\pm0.06$* | $0.35\pm0.07$ |
| Trapidil 200 mg ($n=7$) | $0.28\pm0.08$* | $0.36\pm0.14$ |

Trapidil (50–200 mg) or its vehicle (saline) was injected into the dilated vessel wall of the right femoral artery. After 3 weeks intima or media areas were measured morphometrically in histological sections. *$P<0.05$ when compared to vehicle.

## Results

### *Intima to media area ratio* in vivo

Histological evaluation of the arteries and determination of intimal hyperplasia are shown for the 2 week group in Figure 1a and the 3 week group in Figure 1b, respectively. Trapidil administration resulted in an approximately 60% reduction of the intima to media area ratio (a typical histological example is depicted in Figure 2b) when compared with arteries from vehicle animals (Figure 2a). The reduction of intimal hyperplasia was due to a decreased formation of the intima area as shown in Table 1. Trapidil at the doses used (50–200 mg) was without effect on the media area (Table 1).

### *Bromo-deoxyuridine (BrdU)-incorporation* in vivo

Balloon injury resulted in an increase in the immunostaining of BrdU-positive cells indicating significant proliferation compared to control vessels. Two weeks after angioplasty, administration of trapidil had significantly reduced cell proliferation (a typical histological example is depicted in Figure 3b) in comparison with vehicle treated rabbits (Figure 3a). Values of the degree of proliferation are expressed as % ratio of BrdU-positive cells to total cell number: control (0%, $n=6$), vehicle ($14\pm2\%$, $n=9$ slides from three different animals), vs trapidil 100 mg ($6\pm1\%$, $n=21$ slides from three different animals), *$P<0.05$. In addition, we have determined separately the BrdU-positive cells in the intima and media area of the respective vessels. Values are expressed as % ratio of BrdU-positive cells in

the intima area to total BrdU-positive cells in the intima and media area: control (0%, $n=6$), vehicle ($80\pm3\%$, $n=9$ slides from three different animals) vs trapidil 100 mg ($63\pm9\%$, $n=21$ slides from three different animals). The difference among the latter data was not statistically significant.

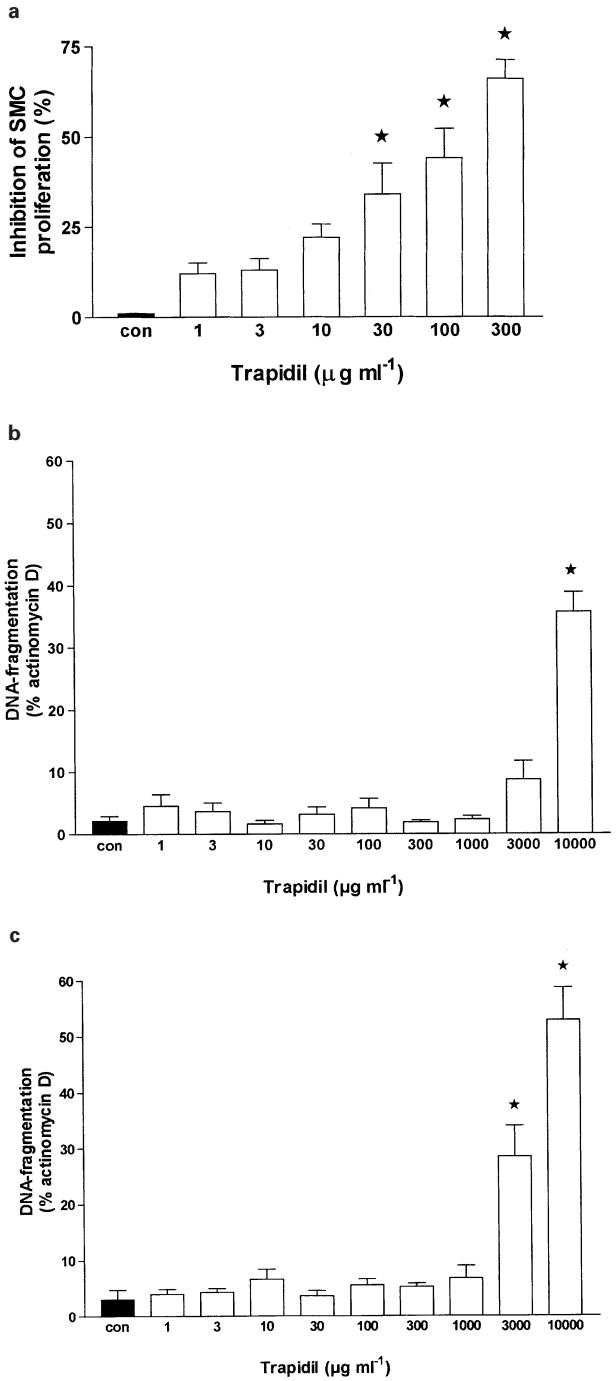


**Figure 4** (a) Effect of trapidil ($1-300$ $\mu$g ml$^{-1}$) on SMC proliferation. Depicted are the calculated inhibitions of SMC proliferation compared to vehicle (control), which has been set at 0% inhibition. Trapidil significantly reduced in a concentration-dependent manner the cell proliferation *in vitro* compared to control ($n=6$ for each group). (b) Effect of trapidil treatment for 24 h ($1-300$ $\mu$g ml$^{-1}$ and $1-10$ mg ml$^{-1}$) on the degree of DNA-fragmentation. All values are expressed as % of DNA-fragmentation induced by actinomycin D. (c) Effect of trapidil treatment for 72 h ($1-300$ $\mu$g ml$^{-1}$ and $1-10$ mg ml$^{-1}$) on the degree of DNA-fragmentation. All values are expressed as % of DNA-fragmentation induced by actinomycin D. (*$P<0.05$ when compared to vehicle).

### *Degree of apoptosis* in vivo

The degree of apoptosis measured using either TUNEL or Hoe 33342 was less than 1% in all groups studied (data not shown). It should be noted that both techniques were able to detect apoptotic cells in histological sections of the following tissues used as positive controls: mammary gland (rat 3–5 days after weaning of pups), thymus and bone marrow from the rabbit (data not shown).

### *SMC proliferation and apoptosis* in vitro

SMC proliferation was FCS dependent (data not shown) and co-incubation with trapidil ($1-300$ $\mu$g ml$^{-1}$) resulted in a significant reduction in SMC proliferation in a concentration-dependent manner, see Figure 4a.

Administration of actinomycin D to SMC cultures induced apoptosis in a concentration dependent manner (data not shown). Induction of apoptosis was time-dependent (Figure 4b, c). When compared to vehicle treated cells, trapidil increased the rate of apoptosis only at the highest concentrations used (3 and 10 mg ml$^{-1}$ after 24 h: Figure 4b and after 72 h: Figure 4c, *$P<0.05$).

## Discussion

New and possible innovative strategies to reduce restenosis after balloon angioplasty involve either the local administration of high doses of therapeutics agents into the coronary vessel wall during balloon angioplasty or local gene therapeutic interventions (Darius *et al*., 1997; Mazur *et al*., 1994; Morishita *et al*., 1993). Numerous catheter devices have been developed in order to enable the local application of DNA, antisense oligonucleotides or drugs (Darius *et al*., 1997; Mazur *et al*., 1994; Morishita *et al*., 1993). Additionally, different techniques are being developed to guarantee a steady release of locally administered drugs, e.g. from drug containing liposomes or microcarriers (Darius *et al*., 1997).

This paper is the first to report the effects of local administration of the functional PDGF antagonist trapidil into the dilated vessel wall of rabbit femoral arteries. In particular, we have demonstrated that trapidil administration at therapeutic doses (i) reduces neointima hyperplasia, i.e. 2 or 3 weeks after balloon angioplasty, (ii) prevents SMC proliferation *in vitro*, (iii) prevents the proliferation of cells with SMC like histological appearance *in vivo*, and (iv) does not induce apoptosis *in vivo* and *in vitro*.

One of the most important problems in clinical cardiology is the development of a restenosis following coronary balloon angioplasty. The exact mechanisms involved are still unresolved but may be due arterial renarrowing which results from both intimal hyperplasia and arterial remodeling (Birnbaum *et al*., 1997; Post *et al*., 1994). Although arterial renarrowing after angioplasty has been attributed largely to intimal hyperplasia, current studies have now demonstrated that arterial remodeling plays a major role in the pathophysiology of restenosis (Birnbaum *et al*., 1997; Post *et al*., 1994). However, in our study we have focused on the effects of local administration of trapidil on intimal hyperplasia following balloon angioplasty.

The morphology of the stenosis after balloon injury is different from that caused by atherosclerosis, as the SMC

change from a contractile to a proliferative status. The timeframe and intracellular signal transduction pathways for development of restenosis have been investigated, but are not entirely clear (Casscells, 1992). Three days after PTCA, growth factors are released by different cells including platelets, SMCs, and fibroblasts, which stimulate the proliferation of SMCs. Most of the growth factors are peptides, which cause mitogenesis due to receptors with intrinsic tyrosine kinase activity, i.e. endothelium growth factor (EGF) or fibroblast growth factor (FGF) (Libby *et al*., 1992). Thus, cytoplasmic proteins such as raf-1, mos, ste II and MAP-(mitogen activated protein)-kinase-kinase isoforms stimulate and activate MAP-kinase, which plays a crucial role in mitogenesis (Davis, 1993). The MAP kinase cascade can also be activated by stimulation of G-protein-coupled receptors by thromboxane $A_2$ or thrombin *via* a mechanism which involves the generation of diacylglycerol (DAG) and activation of protein kinase C (Davis, 1993). MAP-kinase itself regulates immediate early genes and proto-oncogenes of the nucleous, such as c-fos or c-myc (Davis, 1993).

Our findings confirm that trapidil reduces neointima formation after balloon angioplasty (Liu *et al*., 1990; Ohnishi *et al*., 1981), but the precise mechanisms of this effect are unclear. It has been suggested that the anti-proliferative effects of trapidil are due to its ability to block PDGF receptors (Ohnishi *et al*., 1981; Okamoto *et al*., 1992). The anti-mitogenic effects of trapidil may also be due to a PKA-mediated inhibition of raf-1 kinase, and/or inhibition of MAP-kinase and NF$\kappa$B activation (Zwaka *et al*., 1998).

We demonstrate here, that trapidil reduces cell proliferation *via* a mechanism which does not involve the induction of apoptosis. It has been reported (Perlman *et al*., 1997) that balloon injury leads within 30 min to a high rate of apoptosis in SMC in a rabbit and rat model of angioplasty (70% of the medial layer). The rate decreases to $<1\%$ after 4 h, measured using TUNEL, and by the appearance of condensed chromatin. A value of 70% of apoptotic cells would lead to a total collapse of a vessel and is probably incorrect. Recently, it has been reported that RNA synthesis and splicing interferes with DNA *in situ* and labelling techniques (Kockx *et al*., 1998) and this could be an explanation for the high degree of apoptosis measured by Perlman and colleagues. It should be noted that in our study apoptosis was only measured 2 or 3 weeks after vessel injury. At these time points we only determined a degree of apoptosis less than 1% in all vessel area sections. There were no significant differences within these area sections of the different treated groups studied. The reduction in cell proliferation as measured by a reduced BrdU-incorporation *in vivo* or *in vitro* suggests that the mechanism of action of trapidil involves the inhibition of cell proliferation. In additional experiments using a DNA fragmentation assay we have demonstrated that trapidil does not induce apoptosis at therapeutic doses.

Thus, trapidil is an antimitogenic agent and its local administration reduces neointima formation caused by balloon angioplasty in a dose-dependent manner in the rabbit femoral artery *in vivo*. The mechanism involves inhibition of cell proliferation, and does not appear to involve apoptosis. The method of local delivery of drugs into the vessel wall reported in this study may constitute a clinically suitable approach in the therapy of restenosis using trapidil.

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