The IκB Kinase Inhibitor, NEMO-binding Domain Peptide, for Inhibition of Balloon Injury-induced Neointimal Formation

Grassia: The NBD Peptide Inhibits Neointimal Formation

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Abstract

Objective **-** The activation of the nuclear factor (NF)-κB is a crucial step in the arterial wall's response to injury. The identification and characterization of the NF-κB essential modulator (NEMO)-binding domain (NBD) peptide, that can block the activation of the IκB kinase (IKK) complex, have provided an opportunity to selectively abrogate the inflammation-induced activation of NF-κB. The aim of the present study was to evaluate the effect of the NBD peptide on neointimal formation.

Methods and Results **-** In the rat carotid artery balloon angioplasty model local treatment with the NBD peptide (300 μg/site) significantly reduced the number of proliferating cells at day 7 (by 40%; $P<0.01$) and reduced injury-induced neointimal formation (by 50%; $P<0.001$) at day 14. These effects were associated with a significant reduction of NF-κB activation and monocyte chemotactic protein-1 expression in the carotid arteries of rats treated with the peptide. In addition, the NBD peptide (0.01-1 μM) reduced rat SMC proliferation, migration, and invasion *in vitro*. Similar results were observed in apolipoproteinE$^{-/-}$ mice in which the NBD peptide (150 μg/site) reduced wire-induced neointimal formation at day 28 (by 47%; $P<0.01$).

Conclusions **-** The NBD peptide reduces neointimal formation and SMC proliferation/migration, both effects associated with the inhibition of NF-κB activation.

Key Words: Angioplasty, NEMO binding domain peptide, NF-κB, Pharmacology, Vascular biology.

The transcriptional factor nuclear factor κB (NF-κB) plays a critical role in the pathophysiological processes leading to neointimal formation.[1] Activated NF-κB is detected in human restenotic lesions, vascular smooth muscle cells (SMCs), monocytes, and endothelial cells.[2] Activated NF-κB has also been found in balloon-injured rat carotid arteries and has been associated with neointimal formation and expression of NF-κB regulated genes such as vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α).[3-6] Consistent with its role in vascular injury, blocking NF-κB activation via transfection of adenoviral IκB [7] or NF-κB "decoy" oligodeoxynucleotides, attenuated neointimal formation after balloon injury in animal models.[8] Recently, the first clinical use of a NF-κB decoy at the site of coronary stenting for the prevention of restenosis has been described.[9]

NF-κB activation influences SMC viability and migration/invasion by inducing genes with survival functions [5,10,11] and genes involved in matrix degradation.[12] On the other hand, several inflammatory mediators involved in neointimal hyperplasia (e.g. TNF-α), are able to activate NF-κB in SMCs *in vitro*.[5] These findings link the activation of NF-κB to neointimal formation and to the inflammatory response associated with injury-induced SMC proliferation/migration; thus validating NF-κB as a potential target for the control of neointimal hyperplasia. However, the indispensable role played by NF-κB in many biological processes has raised concern that a complete shutdown of this pathway would have significant detrimental effects on normal cellular function. Instead, drugs that selectively target only the inflammation-induced NF-κB activity would be of greater therapeutic value.

A key step in NF-κB activation is the phosphorylation of IκB proteins by the IκB kinase (IKK) complex (IKKα, IKKβ, and NF-κB essential modulator or NEMO).[13] NEMO regulates the IKK complex activity through its binding to the carboxyl-terminal region of the IKKα and IKKβ, termed NEMO-binding domain (NBD). In this regard, a cell-permeable NBD peptide

has been shown to block the association of NEMO with the IKK complex, inhibiting NF-κB activation and ameliorating inflammatory responses.[14] The potential of this peptide as an anti-inflammatory agent has been demonstrated *in vivo* in various animal models including phorbol ester-induced ear edema and zymosan-induced peritonitis,[14] LPS-induced septic shock,[15] mouse carrageenan-induced paw edema,[16] and in a mouse model of experimental arthritis.[17] Importantly the NBD peptide does not completely inhibit NF-κB activity, suggesting that selective disruption of the interaction of NEMO and IKKβ will most likely leave residual NF-κB activity that might be sufficient to maintain normal cellular processes.[14] Nevertheless, the effects of a highly selective pharmacological inhibition of the proinflammatory IKK activity have not yet been investigated in vascular injury.

Therefore, the aim of the present study was to investigate the effect of the NBD peptide on neointimal formation *in vivo* using two well-known animal models of arterial injury: the rat carotid artery balloon angioplasty and the wire-induced carotid injury in apolipoprotein E-deficient ($apoE^{-/-}$) mice. In addition, the effects of the NBD peptide on SMC proliferation and migration *in vitro* were also examined. Our results support the selective targeting of IKK as a powerful approach in the control of neointimal formation.

Methods

Cell culture

Primary aortic SMCs were isolated from the thoracic aorta of male Wistar rats as previously described [18] and grown in Dulbecco's modified Eagle medium (DMEM; Cambrex Bio Sciences) supplemented with L-glutamine, 10% fetal bovine serum (FBS; Cambrex Bio Sciences), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C in 5% CO_2. Before initiation of the assays, the SMCs were starved into DMEM supplemented with 0.1% FBS for 48 h. Studies were performed with cells at passages 3-6.

Cell proliferation study

SMC proliferation was quantified by the total cell number as previously reported.[18] Briefly, $5x10^3$ cells were seeded onto 48-multiwell plates and allowed to adhere overnight. Starved cells were stimulated with TNF-α (5 ng/mL; R&D Systems), PDGF-BB (10 ng/mL; R&D Systems) or FGF-2 (10 ng/mL; R&D Systems) in the presence or absence of the NBD peptide (0.01-1 μM; Genosphere Biotech). After 72 hours, cells were fixed with methanol and stained with Diff-Quik. Proliferation was evaluated as cell duplication by counting the number of cells in ten random fields of each well at 200X magnification (TNF-α experiment) or 400X magnification (PDGF-BB and FGF-2 experiments) with the aid of a 21 mm^2 ocular grid.

Chemotactic migration and invasion

The modified Boyden chamber (48-multiwell plates; Neuroprobe) was used for chemotaxis studies.[18,19] Polyvinyl-pyrrolidone-free polycarbonate filters, 8 μm pore size, were coated with 100 μg/mL collagen type I and 10 μg/mL fibronectin. Biocoat Matrigel invasion chambers (24-multiwell plates with 8.0 μm pore size filter; Becton Dickinson) were used according to the manufacturer's instructions for invasion studies. TNF-α (5 ng/mL), PDGF-BB (10 ng/mL) or FGF-2 (10 ng/mL) were added to the lower wells, while starved cells ($12x10^3$ for migration assay and $3x10^4$ for invasion assay) were seeded into the upper wells of the chamber, and incubated at 37°C. The NBD peptide (0.01-1 μM) was added to the cell suspension 60 min before seeding. After 4h for migration assay or 48h for invasion assay, the migrated cells were fixed and stained with haematoxylin. Cell migration was measured by microscopic evaluation of the number of cells moved across the filter, in ten random fields for the migration assay and in the entire filter for the invasion assay.

Flow cytometry

Apoptosis was quantified by flow cytometry, using a commercially available Annexin V-Alexa Fluor® 488 apoptosis detection kit following the manufacturer's guidelines (Molecular

Probes™). Starved SMCs were stimulated for 24h with TNF-α (5 ng/mL) then washed twice in PBS, trypsinized, and collected. To evaluate the effect of the NBD peptide, SMCs were pretreated for 1h with the peptide (1 μM) before the TNF-α stimulation. Cells were centrifuged, the supernatant discarded, and the cell pellet resuspended in the kit's binding buffer. The cells were centrifuged again, the supernatant discarded, and the pellet resuspended in the kit's buffer containing Alexa Fluor® 488 Annexin V solution and MitoTracker® Red dye. Samples were incubated in the dark for 10 minutes and analyzed using an Epics XL flow cytometer (Beckman Coulter) equipped with a 488-nm Argon laser. Apoptotic cells showed green fluorescence with decreased red fluorescence and live cells showed very little green fluorescence and bright red fluorescence. Isotype-matched antibodies were used as a negative control.

Gelatin zymography

Cells were cultured in 96-well culture plates in 10% FBS medium until 90% confluence was achieved. Starved cells were stimulated with TNF-α (5 ng/mL) in the presence or absence of the NBD peptide (1 μM). After 24h the media were collected, clarified by centrifugation and subjected to electrophoresis in 8% SDS-PAGE containing 1 mg/mL gelatin under non-denaturing conditions. After electrophoresis the gels were washed with 2.5% Triton X-100 to remove SDS and incubated for 24 h at 37°C in 50 mM Tris buffer containing 200 mM NaCl and 20 mM $CaCl_2$, pH 7.4. The gels were stained with 0.5% Coomassie brilliant blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 45% methanol. Bands of gelatinase activity appeared as transparent areas against a blue background. Gelatinase activity was then evaluated by quantitative densitometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were used after the induction of quiescence in 24-well plastic culture plates at a density of 2×10^4 cells/well. The cells were stimulated with TNF-α (5 ng/mL) in the presence or

absence of the NBD peptide (0.01-1 μM). After 24 hours media were collected, centrifuged at 2000xg for 15 min at 4°C and supernatants were used for ELISA to detect MCP-1 (OptEIA^{TM}, Biosciences).

Cytosolic and nuclear extracts

Cells (1×10^5) suspended in 10% FBS medium were seeded in 6-multiwell plates and allowed to adhere overnight. Cells were kept in starving conditions for 48 h. The medium was then removed and replaced with fresh medium containing TNF-α (5 ng/mL) or PDGF-BB (10 ng/mL) in the presence or absence of the NBD peptide (0.01-1 μM) or the mut-NBD peptide (1 μM). The NBD peptides used in this study were described previously.[16]

The cell pellet was resuspended in 100 μL of ice-cold hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatinA, 5 μg/mL leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle five times and centrifuged for 10 min at 13.000×g. The supernatant containing the cytosolic fraction was removed and stored at -80°C. The nuclear pellet was resuspended in 30 μL of high salt extraction buffer (20 mM Hepes pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated at 4°C for 30 min with constant agitation. The nuclear extract was then centrifuged for 10 min at 6000×g with the supernatant aliquoted and stored at -80°C. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad).

Western blot analysis

Immunoblotting analysis of phospho-IκBα (Ser32/36) was performed on cytosolic extracts. The samples were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/mL of bromophenol) in a ratio of 1:1, boiled for 3 min and

centrifuged at 1000×g for 5 min. An equivalent protein amount (30 μg) of each sample was electrophoresed in a 10% discontinuous polyacrylamide gel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation for 2 h with 10% milk buffer and then incubated with the primary antibody (mouse anti-phospho-IκBα, 1:1000; Cell Signaling) at 4°C overnight. The membranes were washed three times with 0.01% Tween20 in PBS and then incubated with anti-rabbit or anti-mouse immunoglobulins coupled to peroxidase (1:1000; DAKO). The immunocomplexes were visualized using the ECL chemiluminescence method.

Electrophoretic mobility shift assay (EMSA)

Double stranded NF-κB consensus oligonucleotide probe (5' AGC TTC AGA GGG GAC TTT CCG AGA GG 3') was end-labelled with [^{32}P]γ-ATP. Nuclear extracts (10 μg protein from each sample) were incubated for 20 min with radiolabelled oligonucleotides (2.5-5.0x10^4 cpm) in 20 μL reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/μL bovine serum albumin, 10% (v/v) glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.5 x Tris-borate/EDTA at 150 V for 2 h at 4°C. The gels were dried and autoradiographed with intensifying screen at -80°C for 24 hours.

Animals

Male Wistar rats (Harlan Laboratories) weighing 250 g and 8-week-old female apoE$^{-/-}$ mice (Charles River) were used. Animals were housed at the Department of Experimental Pharmacology, University of Naples Federico II. All procedures were performed according to Italian ministerial authorization (DL 116/92) and European regulations on the protection of animals used for experimental and other scientific purposes.

Rat carotid balloon angioplasty

Rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) (Gellini

International) and xylazine (5 mg/kg) (Sigma). Endothelial denudation of the left carotid artery was performed with a balloon embolectomy catheter (2F, Fogarty, Edwards Lifesciences) according to the procedure validated in our laboratories.[19,20] Immediately after endothelial denudation, 300 μg of the NBD peptide or 300 μg of the mut-NBD peptide in 100 μL of pluronic gel (pH 7.2) was applied to the adventitia.[21,22] The control group received pluronic gel only. Some animals were subjected to anesthesia and surgical procedure without balloon injury (sham-operated group). Rats were euthanized 7 and 14 days after angioplasty. Carotid arteries were collected and processed as described below.

Atherogenic murine model of vascular injury

ApoE$^{-/-}$ mice were fed an atherogenic diet (21% fat, 0.15% cholesterol, 19.5% casein, wt/wt; TD88137, Mucedola) from 1 week before until 4 weeks after carotid injury performed as described previously,[19] with minor modification. Briefly, mice were anaesthetized as described above, and endothelial injury of the left common carotid artery was performed with a 0.35 mm diameter flexible nylon wire introduced through the left external carotid artery and advanced to the aortic arch. The endothelium was damaged by passing the wire through the lumen of the artery three times. Immediately after endothelial denudation, 150 μg of the NBD peptide or 150 μg of the mut-NBD peptide in 50 μL of pluronic gel was applied to the adventitia. Carotid arteries were collected 28 days after wire injury and processed as described below.

Evaluation of neointimal formation

Carotid arteries were fixed by perfusion with phosphate-buffered saline (PBS; pH 7.2) followed by PBS containing 4% formaldehyde through a cannula placed in the left ventricle. Paraffin-embedded sections were cut (6 μm thick) from the approximate middle portion of the artery and stained with haematoxylin and eosin to demarcate cell types. Ten sections from each carotid artery were reviewed and scored under blind conditions. The cross-sectional

areas of tunica media and neointima were determined by a computerized analysis system (LAS, Leica).

Proliferating Cell Nuclear Antigen Analysis

Proliferating cell nuclear antigen (PCNA) analysis was used to quantify the proliferative activity of cells at the balloon injury sites, and was performed using monoclonal mouse anti-PCNA antibody (1:250, PC10, Sigma) and biotinylated anti-mouse secondary antibody (1:400, DakoCytomation). Slides were treated with streptavidin-HRP (DakoCytomation) and exposed to diaminobenzidine chromogen (DakoCytomation) with hematoxylin counterstain. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blind conditions. Data are represented as percentage of cells positive for PCNA 7 days after angioplasty.

Immunohistochemical localization of the NBD peptide

Localization of the biotinylated (bio)-NBD peptide in rat carotid arteries was performed by immunofluorescence in order to determine the temporal and spatial distribution of the peptide delivered to the adventitia. Briefly, 300 μg of the bio-NBD peptide (Genosphere Biotech, Paris, France) in 100 μl of pluronic gel were applied on the carotid artery immediately after the injury. Immunohistochemical analysis was performed on 5 μm frozen sections of rat carotid artery, 3, 7 and 14 days after injury. The biotinylated peptide was detected by texas red-conjugated streptavidin (1:100; DakoCytomation). For the identification of the SMC a monoclonal anti-α-SMA FITC (1:250, clone 1A4, Sigma) was used. DAPI was used to identify nuclei.

Preparation of Total Extracts of rat carotid arteries

All the extraction procedures were performed on ice with ice-cold reagents as described above. Briefly, liquid nitrogen frozen pooled carotid arteries (n=2) were crushed into powder and resuspended in an adequate volume of hypotonic lysis buffer and then centrifuged for 15

minutes at 6000xg with the supernatant being placed in aliquots, and stored at -80°C. Protein concentration was determined by the Bio-Rad protein assay kit. Total extracts were used to evaluate MCP-1 production and NF-κB activity by ELISA and EMSA respectively.

Statistical analysis

Results are expressed as mean ± SEM of n animals for *in vivo* experiments and mean ± SEM of multiple experiments for *in vitro* assays. Student's *t* test was used to compare 2 groups or ANOVA (Two-Tail *P* value) was used with the Dunnett's *post hoc* test for multiple groups using Graph Pad Instat 3 software (San Diego, CA). Non parametric Mann Whitney test was used for evaluation of neointimal formation. The level of statistical significance was 0.05 per test.

Results

Effect of the NBD peptide on NF-κB activation in SMCs

Thirty minutes of stimulation with TNF-α (5 ng/mL) caused a significant IκBα phosphorylation at Ser32 (Figure 1A). Consistent with its mechanism of action, treatment with the NBD peptide reduced the phosphorylation of IκBα in a concentration-dependent manner. Treatment with the mut-NBD peptide (1 μM) did not affect IκBα phosphorylation (Figure 1A). To further confirm the inhibitory effect of the NBD peptide on NF-κB activation, we examined the NF-κB/DNA binding 4h after TNF-α stimulation. As shown in Figure 1B, the NBD peptide (0.01-1 μM) inhibited TNF-α-induced NF-κB activation. The relative densitometric analysis showed a concentration-dependent inhibition, significant at all concentrations studied (Figure 1B). The NBD peptide alone (1 μM) did not affect NF-κB basal activity (Figure 1B). The mut-NBD peptide (1 μM) showed no effect on TNF-α-induced NF-κB activation (data not shown). The NBD peptide (1 μM) inhibited PDGF-BB (10 ng/mL)-induced NF-κB activation (data not shown).

Effect of the NBD peptide on SMC proliferation and apoptosis

Initiation and maintenance of SMC proliferation is a critical event in the pathogenesis of neointimal formation. As shown in Figure 2A, the NBD peptide (0.01-1 μM) significantly inhibited TNF-α-induced SMC proliferation by 15% ($P<0.05$, n=3), 20% ($P<0.001$, n=3), and 30% ($P<0.001$, n=3) respectively. This effect of the NBD peptide was not due to induction of cell apoptosis as demonstrated by flow cytometry analysis of Annexin V-labelled cells. The NBD peptide (1 μM) neither alone nor in presence of TNF-α (5 ng/mL) stimulated cell apoptosis (Figure 2B). Similarly, the NBD peptide (1 μM) significantly inhibited PDGF-BB (10 ng/mL)-induced SMC proliferation by 27% ($P<0.05$, n=3) but was without effect when the stimulant was FGF-2 (10 ng/mL) (Supplemental Figure IA).

Effect of the NBD peptide on SMC migration

We also evaluated the effects of the NBD peptide on TNF-α-induced SMC chemotaxis. The NBD peptide significantly inhibited chemotactic migration by 15% ($P<0.05$, n=3) at 0.01 μM, and about 20% ($P<0.001$, n=3) at both 0.1 μM and 1 μM (Figure 3A). The NBD peptide reduced PDGF-BB-, but not FGF-2-induced SMC migration (Supplemental Figure IB). Moreover, the NBD peptide (1 μM) significantly reduced SMC TNF-α-induced invasion (by 70%, $P<0.001$, n=3) through the Matrigel™ barrier which mimics the extracellular matrix (Figure 3B).

Effect of the NBD peptide on MMP2 and MMP9 activity

Subconfluent cultures of SMCs were exposed to TNF-α (5 ng/mL) for 24h in the presence or absence of the NBD peptide (1 μM) to assess gelatinase production. Gelatin zymography of control supernatants showed the constitutive release of the latent forms of MMP-2, visualized as a band at 72 and 68 kDa. TNF-α stimulated the release of MMP-2 and induced its activation as revealed by the appearance of the 62 kDa form (Figure 4A). The NBD peptide significantly ($P<0.05$) inhibited the latent form of MMP-2 without affecting the activated

form and slightly decreased, although not significantly, the TNF-α-induced MMP-9 gelatinase active form production (92 kDa) (Figure 4A,B).

Effect of the NBD peptide on MCP-1 production

MCP-1 production by cultured rat SMCs was determined in cell supernatants by ELISA. As shown in Figure 4C, stimulation of SMCs with TNF-α (5 ng/mL) caused an increased release of MCP-1 compared to that observed in unstimulated cells. In the presence of the NBD peptide (0.01-1 μM) a concentration-related inhibition of MCP-1 production was observed. Interestingly, the NBD peptide, at higher concentrations totally abolished TNF-α-induced MCP-1 production. The NBD peptide alone (1 μM) did not affect basal MCP-1 production (Figure 4C).

Effect of the NBD peptide on neointimal formation in rat injured carotid arteries

Rats were treated with either the NBD peptide, the mut-NBD peptide (300 μg/site), or an equal volume of pluronic gel (100 μL, control group) immediately after balloon injury. A reduction of proliferating cells was demonstrated in the carotid arteries of the NBD peptide-treated rats 7 days after injury ($P<0.01$, n=5) (Figure 5A). Moreover, the NBD peptide treatment caused a significant inhibition of neointimal formation by 54% ($P<0.01$, n=10) at day 14 compared with the control group (Figure 5B). The local application of the mut-NBD peptide (300 μg/site) did not affect neointimal formation (n=5; Figure 5B). In addition, the NBD peptide significantly ($P<0.01$) increased the lumen area and decreased neointima/media ratio (Supplemental Table I).

Effect of the NBD peptide on NF-κB activation in rat injured carotid arteries

To support the hypothesis that the reduction of neointimal thickness correlated with NF-κB inhibition, the NF-κB/DNA binding activity was evaluated on extracts from carotid arteries by EMSA. The NBD peptide, but not the mut-NBD peptide, significantly (n=3, $P<0.001$) reduced balloon-induced NF-κB activation in injured arteries 3 and 14 days after injury. A

low level of NF-κB/DNA binding activity was detected in total protein extracts from carotid arteries of sham-operated rats (Figure 5C,D).

Effect of the NBD peptide on MCP-1 production in rat carotid arteries

The NBD peptide was able to significantly inhibit MCP-1 protein production 7, and 14 days after injury, evaluated by ELISA as described above. In the contralateral carotid artery (data not shown) no significant changes were observed at any of the time points, compared with measurements in sham-operated animals (Figure 5E).

Effect of the NBD peptide on neointimal formation in apoE$^{-/-}$ mice

Carotid endothelial denudation was performed in apoE$^{-/-}$ mice fed an atherogenic diet. Twenty-eight days after injury, the neointimal area was reduced by 46% (P<0.01) in apoE$^{-/-}$ mice treated with the NBD peptide compared with mut-NBD-treated mice (Figure 5F). The NBD peptide significantly increased the lumen area (P<0.01) and decreased the neointima/media ratio (P<0.05) (Supplemental Table II).

***In vivo* localization of the bio-NBD peptide**

No positive staining was found in noninjured arteries or pluronic gel-treated carotids 14 days after angioplasty. In contrast, the bio-NBD peptide was detectable in the adventitia and media of injured vessels 3 days following injury. The bio-NBD peptide was also detectable in the media and the neointima at days 7 and 14 (Figure 6).

Discussion

The results obtained in this study show that the local administration of the NBD peptide, a selective inhibitor of IKK activation, reduces neointimal formation in rodent models of vascular injury mainly by inhibiting SMC activation.

Increased SMC proliferation and acquisition of a pro-inflammatory phenotype are central features associated with the development of neointimal lesions.[23] The NBD peptide showed

both *in vivo* and *in vitro* anti-proliferative activity. Treatment with the NBD peptide diminished the number of PCNA-positive proliferating cells in the rat vessel wall 7 days after balloon injury, concomitant with the beginning of neointimal formation. Furthermore, the NBD peptide inhibited *in vitro* TNF-α- and PDGF-BB-induced rat SMC proliferation and migration, effects associated with the inhibition of NF-κB activation. On the contrary, the NBD peptide showed no effect on FGF-2-induced SMC proliferation/migration. This discrepancy could be explained by the fact that FGF-2 induces IκB degradation and NF-κB activation through a pathway distinct from TNF-α,[24] and that p38 and p42/p44 MAPKs are also involved in FGF-2-induced SMC activation.[25] Interestingly, although the NBD peptide significantly reduced rat SMC invasion through the Matrigel barrier, it was able to inhibit only the latent form of MMP2 without significantly affecting the activated forms of both MMP2 and 9, which are known to be required for SMC proliferation and migration into the intimal area of vascular wall.[26,27] These results suggest that other proteases may co-operate with gelatinases in the TNF-α-induced cell invasion process.

Activated NF-κB mediates the expression of several pro-inflammatory genes in SMCs among which, MCP-1 has been demonstrated to play a pivotal role in SMC proliferation/migration [18,28] and neointimal formation in several animal models.[19,28] Interestingly, treatment with the NBD peptide significantly inhibited MCP-1 production, both *in vitro* and *in vivo*. Moreover, the total inhibition of MCP-1 production and SMC proliferation observed at the highest concentration (1μM) suggests that NF-κB triggers the autocrine/paracrine loop mechanism involved in the amplification of the inflammatory vascular response.

Recent findings support the concept of NF-κB as a regional regulator of SMC survival rather than a direct promoter of proliferation of these cells.[10] In our experiments the highest concentration of the NBD peptide showed no effect on SMC apoptosis, either when used alone or with TNF-α. These results are in contrast with previous data obtained by Obara et

al.,[29] showing increased apoptosis rate in TNF-α-stimulated SMCs overexpressing a truncated IκBα. Our results could be justified, at least in part, by the use of a selective inhibitor of the IKK complex formation. It is known that in physiological conditions, NF-κB is partially activated and involved in cell survival.[10] The NBD peptide inhibits only the inflammatory-induced NF-κB activation without modifying the amount constitutively activated.[14,30] To confirm this point we observed that the use of the NBD peptide, at higher concentrations, did not reduce the basal level of MCP-1 production compared to resting cells; most likely reflecting the fact that the NBD peptide does not affect basal NF-κB activity.

In the last ten years, NF-κB has been investigated as a novel therapeutic target to prevent restenosis. However, the potential for developing effective therapeutic strategies based on NF-κB blockade remains to be determined. Several studies have targeted NF-κB activation in the control of vascular injury,[8] and a phase I/IIa open-label multi-center study to assess the inhibitory effects of an NF-κB ODN decoy on in-stent coronary restenosis (INDOR Study) has reported the clinical safety of such an approach in humans.[31]

In contrast to other therapeutic principles, the inhibition of the NF-κB system represents a broad-spectrum, multipurpose-weapon that can interfere with several fundamental pathophysiological mechanisms in the development of neointimal formation; targeting both proliferation and inflammation. In our study, the use of a peptide which selectively inhibits the IKK complex represents a novel and interesting approach. Compared with other NF-κB inhibitors tested to inhibit neointimal formation, the NBD peptide has the advantage of inhibiting the induction of NF-κB activation without inhibiting basal NF-κB activity that may be involved in fundamental cellular processes.[14,30] Notably, the continuous administration of the peptide for >45 days did not lead to overt toxicity in mice.[17]

Importantly, the Pluronic F-127 gel has been shown to be absorbed *in vivo* at 3 days. Such a short-term local administration of traditional NF-κB inhibitors (e.g. pyrrolidine

dithiocarbamate) has been shown to inhibit NF-κB activation in the injured vessels at day 3, without affecting intimal formation at day 14.[6] Intriguingly, using the same delivery approach, we have clearly shown the presence of the biotinylated peptide in the rat media and neointima up to 14 days after injury.

Importantly, the NBD peptide efficacy was also confirmed in the hyperlipidaemic mouse model: demonstrating the efficacy of the peptide under circumstances of increased vascular inflammation.[32]

Both rodent models are not reliable experimental models of human angioplasty and as such, the present study has some limitations. It would be desirable to explore the efficacy and therapeutic potential of the NBD peptide in larger preclinical animal models. However, our results demonstrate the involvement of NF-κB as a regulator in the formation of neointima in mouse and rat vascular injury models and support the use of specific IKK inhibitors to reduce neointimal hyperplasia.

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Disclosure

None.

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Legend to figures

Figure 1. (A) Representative western blot showing the effect of the NBD peptide (0.01-1 μM) or the mut-NBD peptide (1 μM) on rat SMC IκBα phosphorylation induced by TNF-α (5 ng/mL). (B) Representative EMSA and relative densitometric analysis showing the effect of the NBD peptide (0.01-1 μM) on TNF-α (5 ng/mL)-induced NF-κB activation in rat SMCs. Results are expressed as mean ± SEM of 3 separate experiments. ***P<0.001 *vs* TNF-α-stimulated cells.

Figure 2. (A) Upper panel: representative photomicrographs showing the inhibition of TNF-α-induced proliferation by the NBD peptide. In the lower panel the graph shows the effect of the NBD peptide (0.01-1 μM) on SMC proliferation induced by TNF-α (5 ng/mL). Results are expressed as mean ± SEM of 4 experiments run in triplicate. *P<0.05; ***P<0.001 *vs* TNF-α-stimulated cells. (B) Upper panel: representative histograms of apoptotic cells (black) stimulated for 24h with TNF-α (5 ng/mL), and the NBD peptide (1 μM) with or without TNF-α. Positive control: 1mM H_2O_2 for 4h. Isotype-matched antibodies were used as negative control (white). In the lower panel the graph shows the effect of the NBD peptide (1 μM) with or without TNF-α on cell apoptosis. Mean ± SEM of 4 experiments.

Figure 3. (A) Representative photomicrographs showing the inhibition of TNF-α-induced migration by the NBD peptide. In the lower panel the graph shows the effect of the NBD peptide (0.01-1 μM) on SMC migration induced by TNF-α (5 ng/mL). Results are expressed as mean ± SEM of 3 experiments run in triplicate. *P<0.05; ***P<0.001 *vs* TNF-α-stimulated cells. (B) Effect of the NBD peptide on SMC invasion through a matrigel™ barrier induced by TNF-α. Results are expressed as mean ± SEM of 3 experiments run in triplicate. **P<0.01 *vs* TNF-α-stimulated cells.

Figure 4. (A) Representative gel zymography of conditioned medium from SMCs and relative densitometric analysis showing the effect of the NBD peptide on both MMP2 latent

(white columns) and activated (black columns) forms. Results are expressed as mean ± SEM of 4 experiments. *P<0.05 *vs* unstimulated cells; °P<0.05 *vs* TNF-α stimulated cells. (B) Representative gel zymography of conditioned medium from SMCs and relative densitometric analysis showing the effect of the NBD peptide on MMP9 activated form. Results are expressed as mean ± SEM of 4 experiments. **P<0.01 *vs* unstimulated cells. (C) Effect of the NBD peptide on MCP-1 production evaluated by ELISA on SMCs. Results are expressed as mean ± SEM of 3 experiments run in triplicate. **P<0.01 and *** P<0.001 *vs* TNF-α-stimulated cells.

Figure 5. (A) Percentage of total medial and neointimal cells positive for PCNA 7 days after vascular injury. Results are expressed as mean ± SEM (n=5). **P<0.01 *vs* control group. (B) Representative microphotograph showing the effect of local administration of the NBD peptide (300 μg/site; n=10) or the mut- NBD peptide (300 μg/site; n=5) on the neointimal area in rat carotid artery, 14 days after injury. Control animals (n=10) received pluronic gel only as described in Methods. **P<0.001 *vs* control group. Scale bar = 200 μm. (C,D) Representative EMSA and relative densitometric analysis showing the effect of the NBD peptide (300 μg/site; n=3) or the mut-NBD peptide (300 μg/site; n=3) on NF-κB activation in rat carotid arteries 3 (C) and 14 (D) days after angioplasty. Results are expressed as mean ± SEM of 3 separate experiments. ***P<0.001 *vs* control group. (E) Effect of the NBD peptide (300 μg/site) on MCP-1 expression in rat carotid arteries 7 and 14 days after balloon injury evaluated by ELISA. Results are expressed as mean ± SEM of MCP-1 levels normalized with protein concentrations, where n=3. **P<0.01 and ***P<0.001 *vs* control group; °°°P<0.001 *vs* sham-operated animals. Sham (grey columns), Control (black columns), NBD (white columns). (F) Effect of local administration of the NBD peptide (150 μg/site; n=6) on neointimal area in apoE$^{-/-}$ mouse carotid artery, 28 days after injury. **P<0.001 *vs* mut-NBD treated group (n=6). Scale bar = 100 μm.

Figure 6. Immunofluorescence visualization of α-SMA (green) and bio-NBD peptide (red) in rat carotid arteries 3, 7, and 14 days after balloon angioplasty. Dapi (blue) was used to locate nuclei. In the control group was used the pluronic gel only.

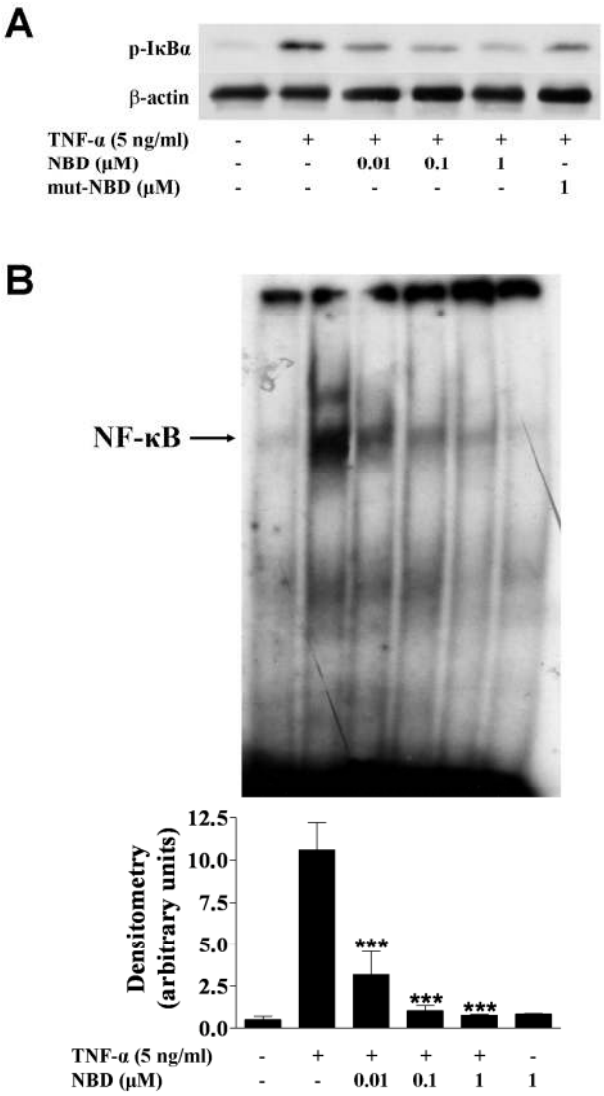
A
p-IκBα
β-actin
TNF-α (5 ng/ml) - + + + + +
NBD (μM) - - 0.01 0.1 1 -
mut-NBD (μM) - - - - - 1
B
NF-κB
Densitometry (arbitrary units)
12.5
10.0
7.5
5.0
2.5
0.0
***
***
***
TNF-α (5 ng/ml) - + + + + -
NBD (μM) - - 0.01 0.1 1 1

Figure 1

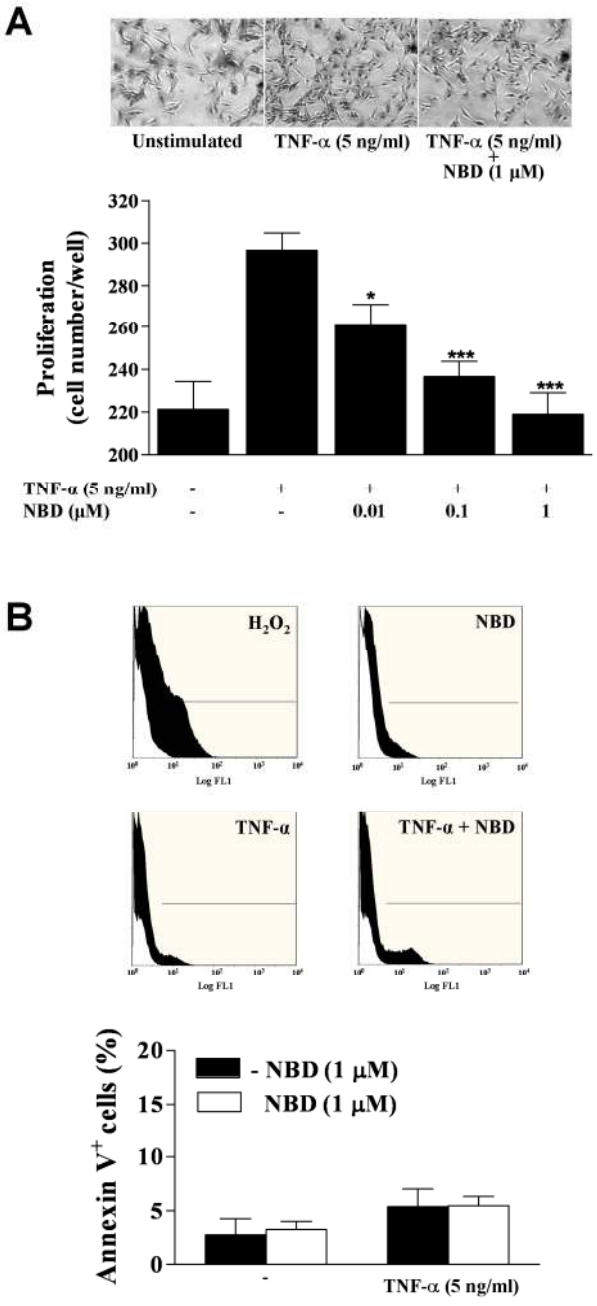
A
Unstimulated
TNF-α (5 ng/ml)
TNF-α (5 ng/ml) + NBD (1 μM)
Proliferation (cell number/well)
320
300
280
260
240
220
200
*
***
***
TNF-α (5 ng/ml) - + + + +
NBD (μM) - - 0.01 0.1 1
B
H2O2
NBD
TNF-α
TNF-α + NBD
Log FL1
Annexin V+ cells (%)
20
15
10
5
0
- NBD (1 μM)
NBD (1 μM)
-
TNF-α (5 ng/ml)

Figure 2

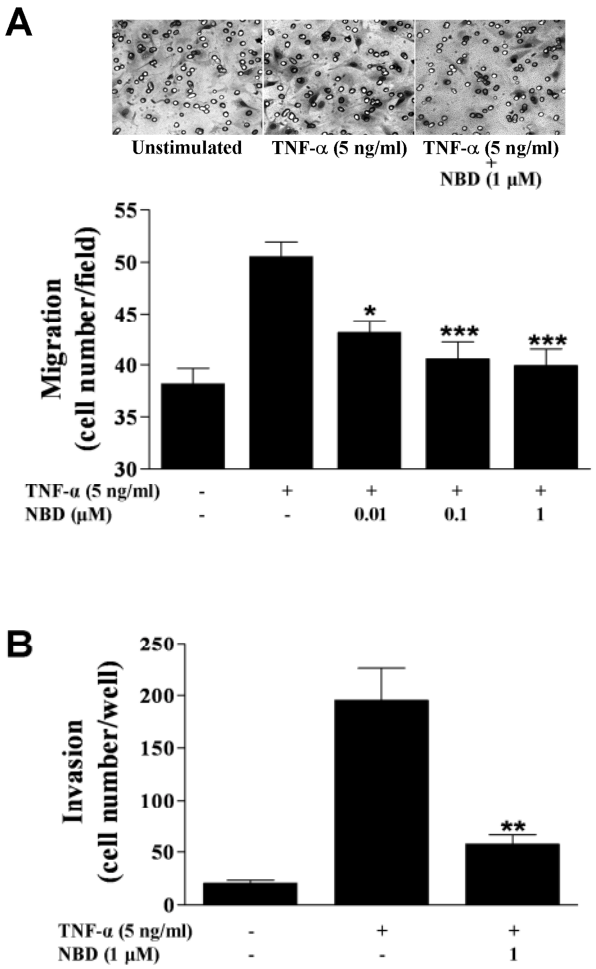
A
Unstimulated
TNF-α (5 ng/ml)
TNF-α (5 ng/ml) + NBD (1 μM)
Migration (cell number/field)
55
50
45
40
35
30
*
***
***
TNF-α (5 ng/ml) - + + + +
NBD (μM) - - 0.01 0.1 1
B
Invasion (cell number/well)
250
200
150
100
50
0
**
TNF-α (5 ng/ml) - + +
NBD (1 μM) - - 1

Figure 3

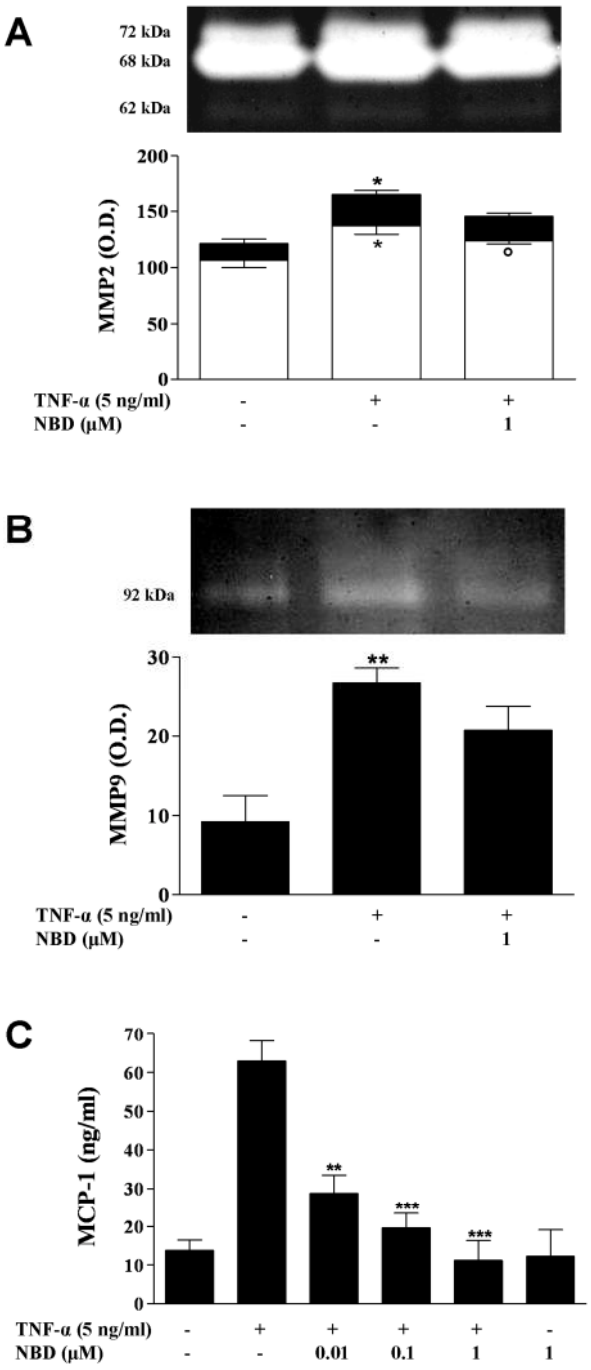
A
72 kDa
68 kDa
62 kDa
MMP2 (O.D.)
200
150
100
50
0
TNF-α (5 ng/ml) - + +
NBD (μM) - - 1
B
92 kDa
MMP9 (O.D.)
30
20
10
0
TNF-α (5 ng/ml) - + +
NBD (μM) - - 1
C
MCP-1 (ng/ml)
70
60
50
40
30
20
10
0
TNF-α (5 ng/ml) - + + + + -
NBD (μM) - - 0.01 0.1 1 1

Figure 4

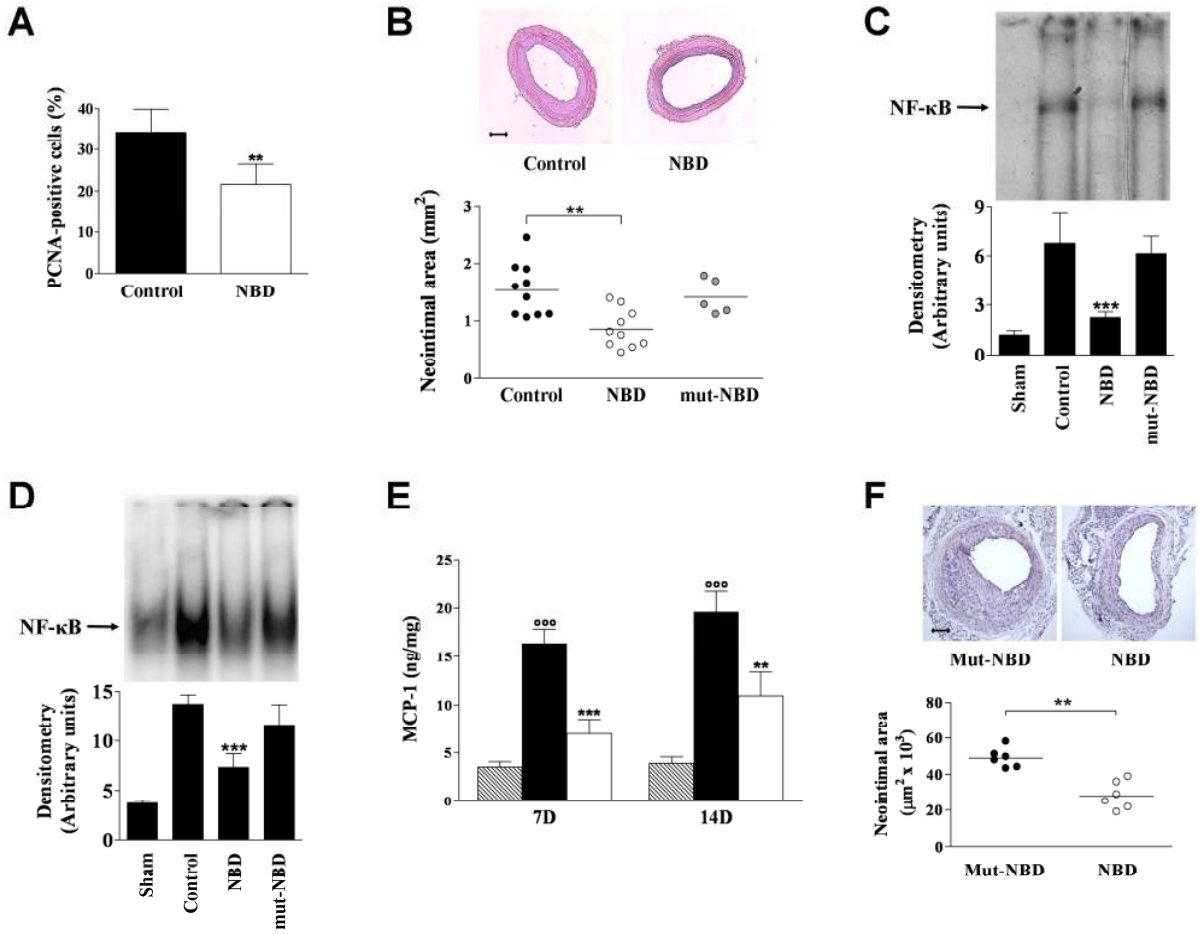
A
PCNA-positive cells (%)
40
30
20
10
0
**
Control
NBD
B
Control
NBD
Neointimal area (mm2)
3
2
1
0
**
Control
NBD
mut-NBD
C
NF-κB
Densitometry (Arbitrary units)
9
6
3
0
***
Sham
Control
NBD
mut-NBD
D
NF-κB
Densitometry (Arbitrary units)
15
10
5
0
***
Sham
Control
NBD
mut-NBD
E
MCP-1 (ng/mg)
25
20
15
10
5
0
ooo
***
ooo
**
7D
14D
F
Mut-NBD
NBD
Neointimal area (μm2 x 10³)
80
60
40
20
0
**
Mut-NBD
NBD

Figure 5

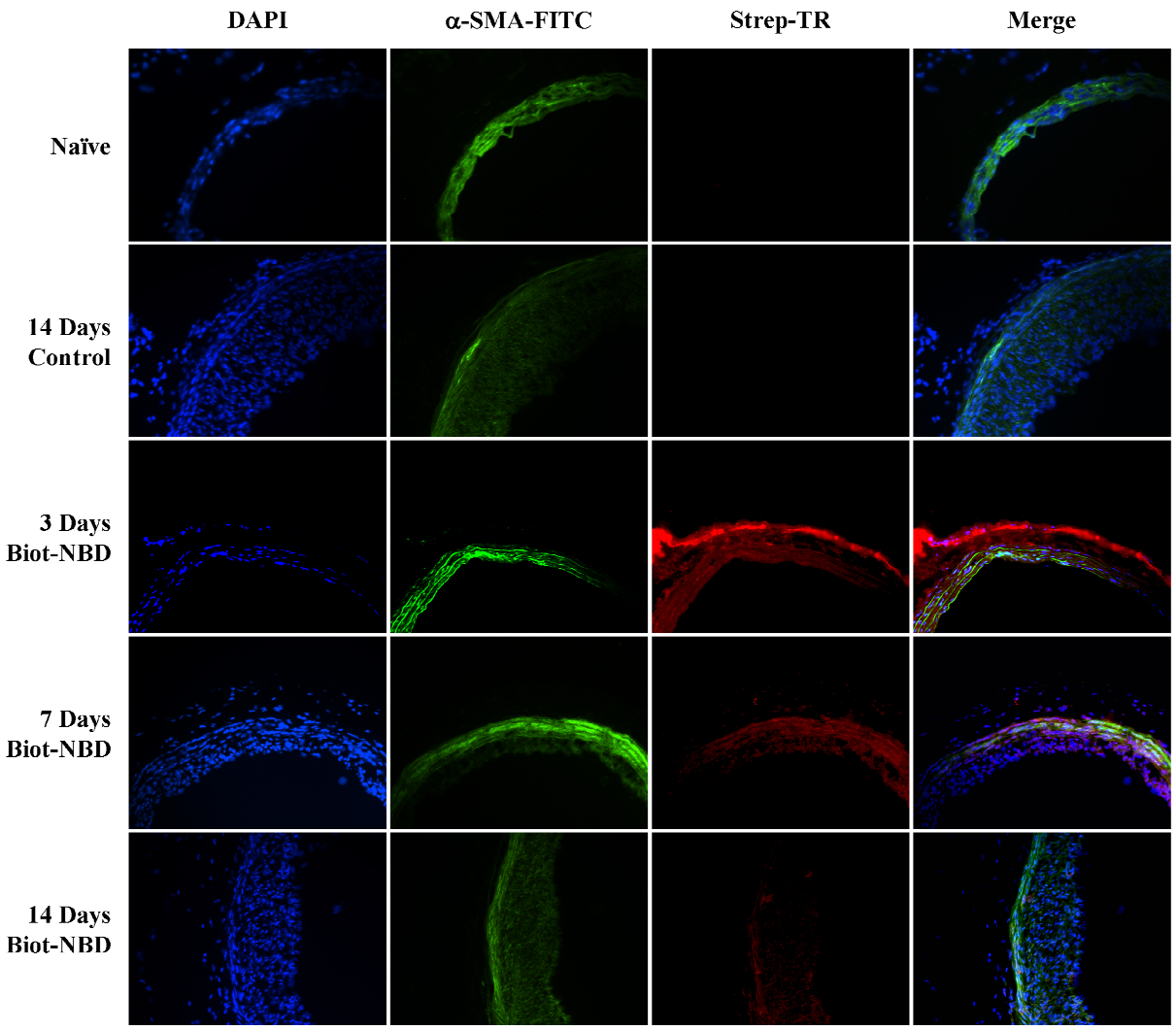
DAPI
α-SMA-FITC
Strep-TR
Merge
Naïve
14 Days Control
3 Days Biot-NBD
7 Days Biot-NBD
14 Days Biot-NBD

Figure 6