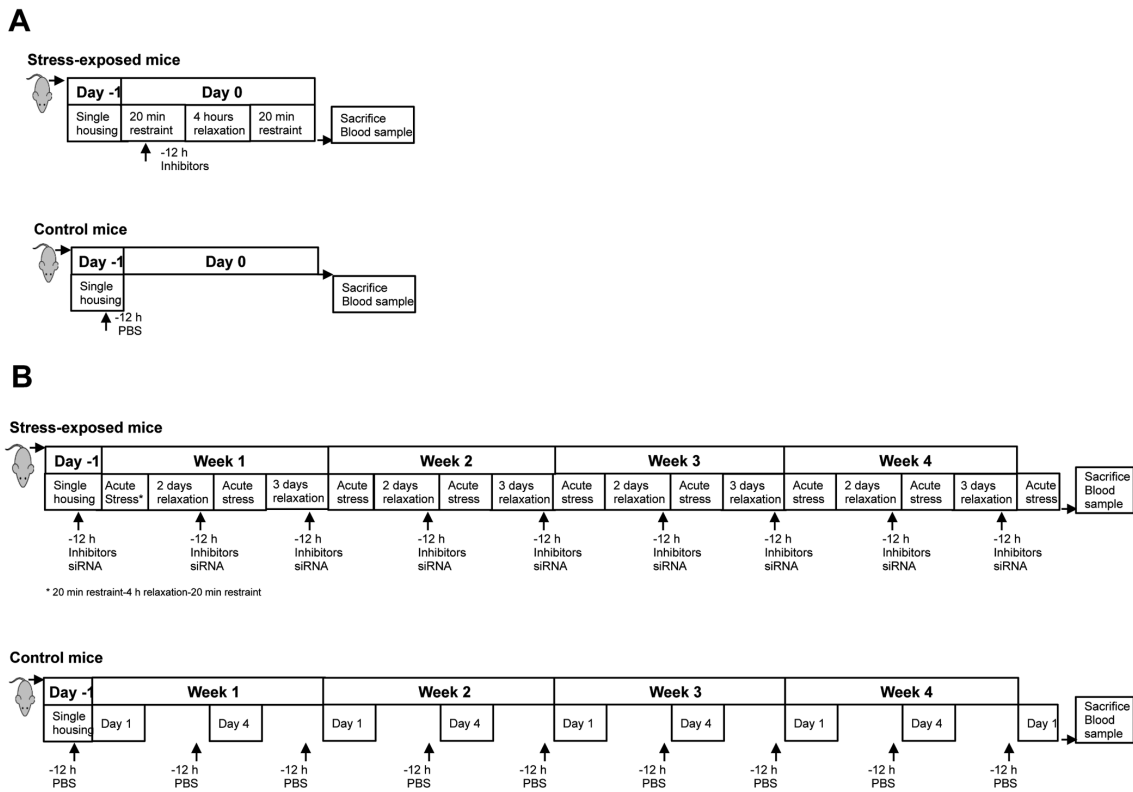


Targeting Activation of Specific NF- κ B Subunits Prevents Stress-Dependent Atherothrombotic Gene Expression

Zdenka Djuric,¹ Muhammed Kashif,¹ Thomas Fleming,¹ Sajjad Muhammad,² David Piel,¹ Rüdiger von Bauer,¹ Florian Bea,³ Stephan Herzig,⁴ Martin Zeier,¹ Marina Pizzi,^{5,6} Berend Isermann,¹ Markus Hecker,⁷ Markus Schwaninger,² Angelika Bierhaus,^{1†} and Peter P Nawroth¹

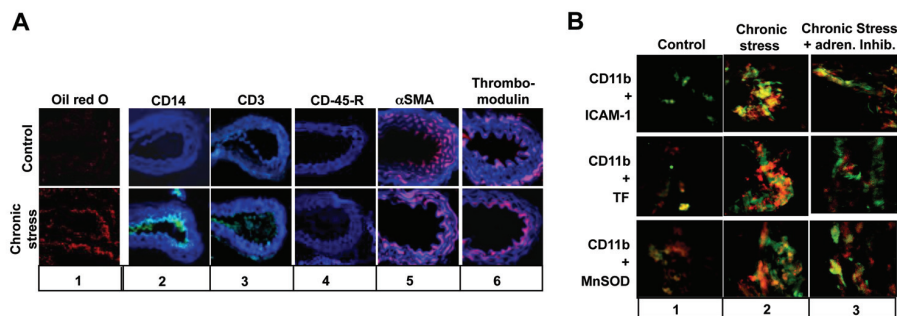
Online address: <http://www.molmed.org>

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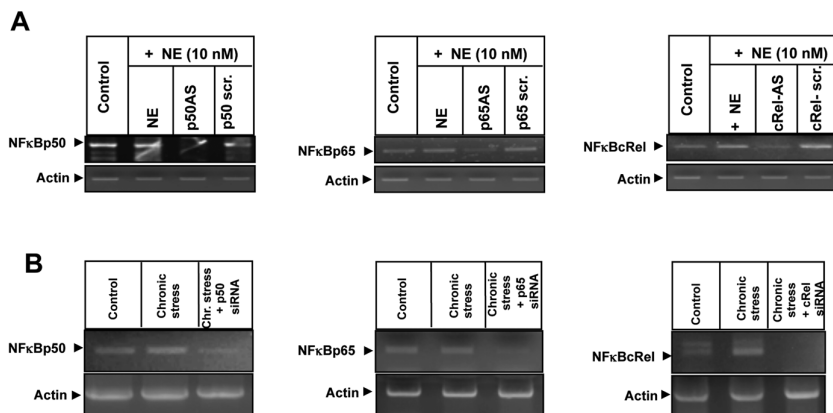


Supplementary Figure S1. Flowchart of the animal experiments. (A) Experimental procedures in the model of acute stress. (B) Experimental procedures in the model of chronic stress.

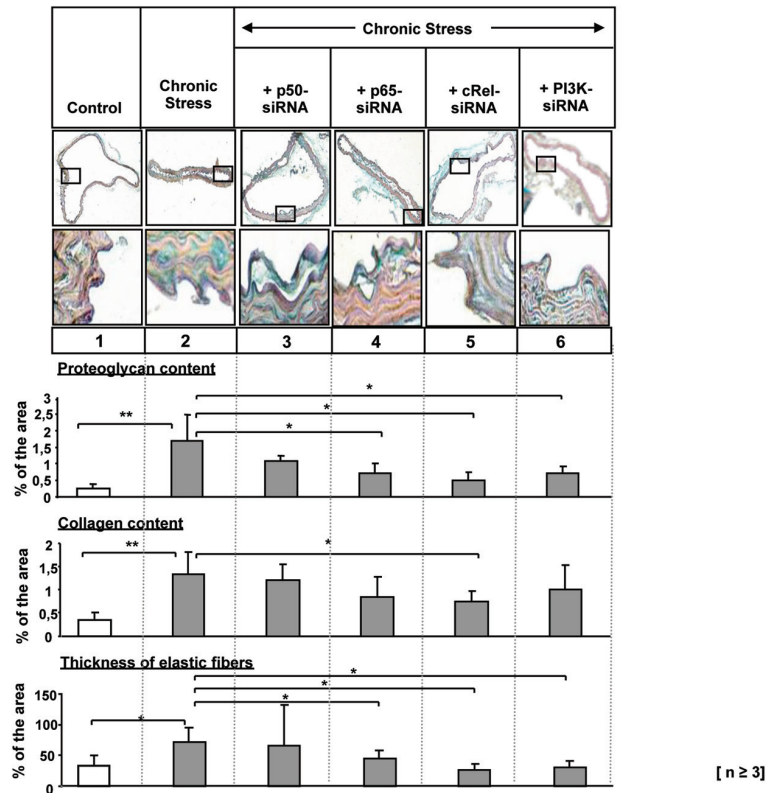
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Supplementary Figure S2. Exposure to transient and repeated restrain stress induces cellular changes in vascular wall. (A) ApoE^{-/-} mice were left untreated or were to repeated stress episodes as described in Materials and Methods. Frozen aortic sections were stained with Oil red O, visualised in bright field, whereas photographs were taken to the dark background afterwards. Sections were stained for the monocyte/macrophage marker CD14, the T-Lymphocyte marker CD3 (lane 3), the B-Lymphocyte marker CD45-R, α - smooth muscle actin (lane 5) and the endothelial marker thrombomodulin. CD14 and CD3 were visualised using FITC-green dye, Texas-Red dye was used for CD45-R, α SMA and thrombomodulin. All sections were counterstained with nuclear stain DAPI and co-localisation has been shown using merged photographs. Magnification: 20x. (B) Transverse frozen aortic sections were co-stained with antibodies for the macrophage marker CD11b and ICAM-1, TF and MnSOD. CD11b is shown by green FITC-labelling. ICAM-1, TF and MnSOD are visualized by Texas Red labelling and co-localisation of the antigens appears in yellow colour (Magnification: 20x).



Supplementary Figure S3. (A) THP-1 cells were preincubated with antisense (as) or scrambled (scr.) ODNs for p50, p65 or cRel for 48h (suppl. Table 3) and thereafter incubated in the absence or presence of NE (10 nM) for 1 h. Down-regulation of p50, p65 and cRel by specific asODNs was confirmed by RT-PCR. The experiment was repeated twice with identical results and one representative experiment is shown. (B) RT-PCR demonstrates efficient down-regulation of p50, p65 and cRel *in vivo* using siRNA.



Supplementary Figure S4. Morphology of advanced lesions in ApoE^{-/-} mice exposed to repeated stress episodes. Frozen, aortic sections were stained with Movat pentachrome stain as described in Materials and Methods. Magnification: 4x, 20x. Sections were analysed with Image Pro software and the content of proteoglycans, collagen and thickness of the elastic fibers of 10 independent stains were evaluated by one investigator blinded to the identities of the experimental groups. Statistically significant differences are indicated by * (* = p < 0.05). Data represent the mean ± SD (n ≥ 3).

Supplementary Table S1. Oligonucleotides used in EMSA and ChIP

TF-NFκB bindingsite (EMSA)	Sense: 5'- ggggtgagtcacccctgcagggtcccggagttctaccgggaggagcg ggg -3' Antisense: 5'-ccccgcctcctcccggtaggaactcgggaccctgcaagggatgact cacccc-3'
ICAM-1-NFκB binding site (EMSA)	Sense: 5'-gccaccgcccggcattgctttagcttggaaattcgggagctgaagcggcc agc-3' Antisense: 5'-gctggccgcttcagctccggaattccaagctaaagcaatcgggcggc ggtgcc-3'
MnSOD-NFκB binding site (EMSA)	Sense: 5'-agactgggaataccccagttgt-3' Antisense: 5'-acaactgggtattccccagtct-3'
ICAM-1-promoter sequence for ChIP	Forward: 5'-gcggtgtagaccgtgattca-3' Reverse: 5'-ctgcagttattccggactgacag-3'
Human TF-promoter sequence for ChIP	Forward: 5'-gcaactagaccgcctgcgtcctc-3' Reverse: 5'-cggccccggcggagtcaca-3'
Human MnSOD-intron-sequence for ChIP	Forward: 5'-cgaaccttgattacgggaaa-3' Reverse: 5'- cctggtgcagatgttgcct-3'
murine ICAM-1 promo-ter sequence for ChIP	Forward: 5'-cattactcagtttggaaattcctagatc-3' Reverse: 5'- ggaacgagggtcctcggtatt-3'

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Supplementary Table S2. Primers used in RT-PCR experiments.

ICAM-1	Forward: 5'-tatggcaacgactccttct-3' Reverse: 5'-cattcagcgtcaccttg-3'
TF	Forward: 5'-caacacttcctaagcctcc-3' Reverse: 5'-aaagttccggtcacagtgc-3'
MnSOD	RT ² PCR primer set for human MnSOD, Acc.Nr. NM_000636, <i>Superarray, Frederick, MD, USA</i>
NF κ Bp50	Forward: 5'-gaccaaggagatggacctca-3' Reverse: 5'-cgaagctggacaaacacaga-3'
NF κ Bp65	Forward: 5'-gactgccggatggcttctatgagg-3' Reverse: 5'-ccgccgcagctgcatggagac-3'
NF κ BcRel	Forward: 5'-gccatctcaagtggattgt-3' Reverse: 5'-tctctccatgctgctactg-3'
actin	Forward: 5'-agaggtatcctgacctgaagtacc-3' Reverse: 5'-ccaccagacaacctgttggcat-3'
murine ICAM-1	Forward: 5'-ttcaactgaatgccagctc-3' Reverse: 5'-gtctgctgagacccctcttg-3'
murine TF	Forward: 5'-gccggataccatcactcgtccctccg-3' Reverse: 5'-ttcctccgtggtagagagggaccttg-3'
murine MnSOD	Forward: 5'-atgtctgtggagtcctca-3' Reverse: 5'-tgaaggtagttagcgtgctc-3'
murine NF κ B p50	Forward: 5'-attgtcttcaaacgccaaagt-3' Reverse: 5'-atggaatgtaatcccaccgtag-3'
murine NF κ B p65	Forward: 5'-gcgtacacattctgggg-3' Reverse: 5'-aggagctccacaggacagaa-3'
murine NF κ B cRel	Forward: 5'-ttaccagaaatgccaggtc-3' Reverse: 5'-aggcccttctaggaatggaa-3'
murine PI3K	Forward: 5'-tgtggacctctgctgtca-3' Reverse: 5'-aggagctccacaggacagaa-3'

Supplementary Table S3. PTO-modified antisense oligonucleotides (asODNs): The position of PTO-modifications is indicated with capital letters.

NF κ Bp50	Antisense: 5'-Caa atatGgatcatcttCtgc caT-3' Scrambled: 5'-Gca aaTtaattcttaCctagc ctG-3'
NF κ Bp65	Antisense: 5'-Gat gag Gggaac act tCgtctaC-3' Scrambled: 5'-GggtacgaCtcagaGaaactgttG-3'
NF κ BcRel	Antisense: 5'-Cgg gttataCgcaccGgaggc caT-3' Scrambled: 5'-Gtt ataacCggaCgg cat cgc ggC-3'

Supplementary Table S4. siRNA-Sequences.

Non-specific siRNA	5'- agguaguguaaucgccuugt-3'
human MnSODsiRNA	5'- ggaaccucacaucaacgca - 3'
murine MnSODsiRNA	5'- aucuauuuuaggaacuaatt-3'
murine NF κ B p50 siRNA	5'- gcagguuuuagacauacuatt - 3'
murine NF κ B p65 siRNA	5'- ggcgauuggcuuuacuua-3'
murine NF κ B cRelsiRNA	5'- gaauuacauuaguacaaa-3'
murine PI3K siRNA	5'- caacuuaauuacuuaatt -3'

Supplementary Table S5. RT-PCR profiling arrays for the expression of genes involved in antioxidant defence, NF κ B signaling and atherosclerosis.

List of the genes included in all three profiling arrays which are suppressed by NF κ B p50 antisense oligonucleotide	List of the genes included in all three profiling arrays which are suppressed by NF κ BcRel antisense oligonucleotide
<p>Albumin, 12-lipoxygenase, Angiopoietin-like 7, Aldehyde oxidase 1, ATX1 antioxidant protein 1 homolog, BCL2/adenovirus E1B protein 3, Copper chaperone for SOD, Cold shock domain containing E1, Diacylglycerol kinase, Dual oxidase 1, Dual oxidase 2, Dual specificity phosphatase, Epoxidehydrolase 2, Eosinophil peroxidase, Forkhead box M1, Glutaredoxin 2, GPCR 156, GPx2, GPx3, GPx5, GPx6, GPx7, Glutathione reductase, Glutathione synthetase, Glutathione transferase zeta 1, General transcription factor II, Keratin1 Lactoperoxidase, Mannose-binding lectin, Myeloperoxidase, MpV17 mitochondrial inner membrane protein, Methioninesulfoxidereductase A, Metallothionein 3, Metallothionein-like 5, NCF-1, NCF-2, Non-metastatic cells 5 protein, iNOS, NADPH oxidase, Nudix-type motif 1, Oxidation resistance 1, OSR-1, Phosphoinositide-binding protein PIP3-E, Peroxiredoxin 1, Peroxiredoxin 2, Peroxiredoxin 3, Proteoglycan 3, Prion protein, COX-1, COX-2, Peroxidase homolog, Peroxidase homolog-like, Scavenger receptor class A member 3, Selenoprotein S, Selenoprotein P, Serum/glucocorticoid regulated kinase 2, Cu/ZnSOD, MnSOD, EC-SOD, Sulfiredoxin 1, Thyroid peroxidase, Titin, Thioredoxin domain containing 2, Thioredoxin reductase 1, Activating transcription factor 1, BIAP-containing 2, CASP1, CASP8 and FADD-like apoptosis regulator, IKKα, Gpcr26/LPA1, Thrombin receptor, Gap junction protein α1, 5-HT2B, ICAM-1, IFNβ, IKKβ, IKKγ, IL-10, IL-1α, IL-1β, IL-6, LTα, CD18, MLT-translocation gene 1, MEKK, NF-κBp50/p105, IKBα, NF-κBp65, Ras homolog gene family member A, TLR-1, CD27, TLR-7, TLR-8, TLR-9, TNFα, TNFS10b, TNFS10, ACE-1, BCL2-related protein A1, BCL2-like 1, BH3 interacting domain death agonist, BIAP-containing 3, CSF-2, Egr-1, elastin, endoglin, FABP3, Heparin-binding EGF-like growth factor, Fas, FGF-2, IFNR, IL-1RB, CD11c, Kruppel-like factor 2, LDLR, Lipoprotein lipase, MMP-1, eNOS, Nuclear receptor subfamily 1H3, PDGFβ, PPARα, Retinoid X Receptor, PAI-2, Cu/ZnSOD, BSP-1, TGFβ2, TSP-4, Tenascin C, TNFα-induced protein 3, VEGFA, vWF</p>	<p>Albumin, 12-lipoxygenase, 24-dehydrocholesterol reductase, Diacylglycerol kinase, Dual oxidase-1, GPx2, Glutathione reductase, Glutathione synthetase, Glutathione transferase zeta-1, Keratin-1, iNOS, NCF-1, Eln, Peroxiredoxin 1, Prion protein, Scavenger receptor class A member 3, Selenoprotein S, Sirtuin, Cu/ZnSOD, Titin, Thioredoxin reductase 1, ATF-1, B-cell CLL/lymphoma 10, B-cell CLL/lymphoma 3, BIAP repeat-containing 2, Caspase-8, CCL-2, CASP-8 and FADD-like apoptosis regulator, IKKα, CSF-2, CSF-3, Gpcr26/LPA1, EDAR-associated death domain, Thrombin receptor, Fas-associated via death domain, Gap junction protein α1, 5-HT2B, ICAM-1, IFNβ1, IKKα, IL-10, IL-1α, IL-6, IL-8, IRAK-1, CD18, MEKK, NF-κBp50/p105, MLT-translocation gene-1, TNFS10, TLR-4, ACE-1, ApoB, ApoE, BAX, BCL2-like 1, BCL2-related protein A1, BH3 interacting domain death agonist, BIAP-containing 3, CCL-5, CCR-2, VE-cadherin, Egr-1, elastin, endoglin, CTGF, FABP-3, Fas, FGF-2, INFR-2, IL-1R1, IL-1R2, IL-3, IL-5, CD49e, CD11c, Kruppel-like factor 2, LDLR, MMP-1, eNOS, PPARα, Nuclear receptor subfamily 1H3, COX-1, Retinoid X Receptor, CD162, PAI-2, TGFβ2, BSP-1, Thrombospondin 4, Tenascin C, TNFα-induced protein 3, VCAM-1, VEGFA, vWF</p>