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Cezanne regulates inflammatory responses to hypoxia in endothelial cells by targeting TRAF6 for deubiquitination

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Abstract

Rationale—Hypoxia followed by reoxygenation promotes inflammation by activating NF-κB transcription factors in endothelial cells (EC). This process involves modification of the signalling intermediary TRAF6 with polyubiquitin chains. Thus cellular mechanisms that suppress TRAF6 ubiquitination are potential therapeutic targets to reduce inflammation in hypoxic tissues.

Objective—In this study, we tested the hypothesis that endothelial activation in response to hypoxia-reoxygenation can be influenced by Cezanne, a deubiquitinating enzyme that cleaves ubiquitin from specific modified proteins.

Methods and Results—Studies of cultured endothelial cells (EC) demonstrated that hypoxia (1% oxygen) induced Cezanne via p38 MAP kinase-dependent transcriptional and post-transcriptional mechanisms. Hypoxia-reoxygenation had minimal effects on pro-inflammatory signalling in unmanipulated EC but significantly enhanced Lys-63 polyubiquitination of TRAF6, activation of NF-κB and expression of inflammatory genes following silencing of Cezanne. Thus although hypoxia primed cells for inflammatory activation it simultaneously induced Cezanne which impeded signalling to NF-κB by suppressing TRAF6 ubiquitination. Similarly, ischemia induced Cezanne in the murine kidney in vascular EC, glomerular EC, podocytes and epithelial

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cells, and genetic deletion of Cezanne enhanced renal inflammation and injury in murine kidneys exposed to ischemia followed by reperfusion.

Conclusions—We conclude that inflammatory responses to ischemia are controlled by a balance between ubiquitination and deubiquitination and that Cezanne is a key regulator of this process. Our observations have important implications for therapeutic targeting of inflammation and injury during ischemia-reperfusion.

Keywords

Endothelium; hypoxia; inflammatory activation

Introduction

Tissues are exposed to hypoxia followed by reoxygenation during ischemia-reperfusion which occurs in several clinical settings including organ transplantation, percutaneous coronary intervention and bypass grafting[1,2]. Hypoxia-reoxygenation promotes inflammation by activating NF-κB transcription factors which induce adhesion proteins (e.g. VCAM-1, E-selectin) and other inflammatory molecules[3–9]. NF-κB transcription factors are regulated by an intricate signalling network that governs their intracellular localisation and transcriptional activity[10]. In the basal state, NF-κB dimers are inactivated by binding to inhibitor of κB (IκB) proteins which sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. IκB kinase (IKK) promotes NF-κB activation by phosphorylating IκBα, a modification that targets it for ubiquitin- mediated degradation, and by phosphorylating RelA NF-κB subunits to enhance DNA binding and transcriptional activation[11].

Recent studies have shed light on the signalling events that control NF-κB activation by hypoxia. In normoxic conditions, IKK proteins are targeted for hydroxylation by prolyl hydroxylase domain 1 (PHD1), a modification that leads to their ubiquitination and degradation[4]. Hypoxia can promote nuclear localization of NF-κB by preventing PHD1-mediated repression of IKK[4], and by inducing the expression of several NF-κB sub-units[6]. The mechanism of NF-κB activation by hypoxia also involves modification of TRAF6 proteins with a non-canonical form of polyubiquitin (linked through Lys63) that is known to activate IKK[12,13]. Despite these insights, the mechanisms for NF-κB activation by hypoxia are understood incompletely and may vary according to particular cell types.

We previously discovered a regulator of NF-κB called Cezanne (Cellular Zinc-finger Anti-NF-κB; also known as OTUD7B) and cloned full length cDNA from EC[14]. Our subsequent studies revealed that Cezanne can be induced by TNFα, IL-1 and functions as an inhibitor of NF-κB, thus forming a negative feedback loop in inflammatory cytokine signalling[15,16], and a recent report indicated that Cezanne can also influence non-canonical NF-κB activation[17]. The molecular mechanism for the anti-inflammatory effects of Cezanne was recently illuminated by our group who demonstrated that this molecule belongs to a novel family of deubiquitinating enzymes[18]. Thus Cezanne suppresses activation of NF-κB in response to TNFα by cleaving polyubiquitin chains from RIP1 which is a component of the TNFR complex[15,16]. Cezanne is related to a protein called A20 (TNFAIP3) which can also

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suppress NF-κB activation via deubiquitination of signalling proteins[19,20]. However, although A20 and Cezanne possess partially overlapping biochemical properties, they exert unique functions by targeting distinct forms of polyubiquitin[20,21]. Here, we examined whether Cezanne can influence inflammatory responses to hypoxia-reoxygenation in EC using both *in vitro* and *in vivo* models.

Methods

Reagents and antibodies

Anti-Cezanne (Proteintech Europe Ltd), Anti-RelA (p65), anti-κBα, anti-TRAF6, anti-Lamin B (Santa Cruz Biotechnology), phosphorylated anti-ATF2 (Thr71), phosphorylated anti-RelA (Ser536) (Cell Signalling Technology), anti-ubiquitin (Invitrogen), anti- Lys63 polyubiquitin, anti-GAPDH (Merck-Millipore), anti-α-tubulin (Sigma-Aldrich) anti-kidney injury marker-1 (R&D Systems) and polyclonal goat anti-rabbit and anti-mouse conjugated horse radish peroxidise (HRP) (Dako) antibodies were obtained commercially. Rabbit anti-Cezanne polyclonal antibodies were raised and subsequently affinity purified as described[15]. Pharmacological inhibitors of p38 (CT8730, UCB Celltech & Sb202190, Merck Chemical) and actinomycin D (Sigma-Aldrich) were obtained commercially and dissolved in DMSO.

Endothelial cells and exposure to hypoxia

Human coronary microvascular EC (HCMEC) and human dermal microvascular EC (HDMEC) were obtained commercially (Promocell). Human umbilical vein endothelial cells (HUVEC) were collected using collagenase. EC were cultured as described previously[15]. Confluent HUVEC cultures were exposed to 1% - 5% O_2 using the Ruskinn Hypoxia Workstation prior to re-oxygenation (20% O2; normoxia) as described[9]. Hypoxia is defined as exposure of cells to 1% O_2 unless stated otherwise.

RNA interference

Cell cultures were transfected with siRNA sequences that are known to silence Cezanne (5'-GAAUCUAUCUGCCUUUGGA-3'; Dharmacon[14] or SMARTpool ON- TARGETplus Human Otud7b siRNA), ATF2 (Dharmacon or SMARTpool ON-TARGETplus Human ATF2 siRNA) or p38α (Dharmacon) using the Neon transfection system (Life Technologies) and were then incubated in antibiotic-free growth medium for 48 h before analysis. Alternatively, they were transfected with non-targeting scrambled controls (Silencer Negative control # 1 siRNA; Ambion). To control for the possible effects of mRNA processing, cells were transfected with siRNA targeting SHP2 (Dharmacon) which is unlikely to be involved in TRAF signalling.

Comparative real time PCR

RNA was extracted using the EZNA Total RNA Kit I (Omega Bio- tek) and reverse transcribed into cDNA using qScript cDNA Supermix (Quanta Biosciences). Transcript levels were quantified by comparative real-time PCR using gene-specific primers for human Cezanne (sense, 5'-ACAATGTCCGATTGGCCAGT-3'; antisense, 5'-ACAGTGGGATCCACTtCaCATTC-3'), human E-selectin (sense, 5'-GCTCTGCAGCTCGGACAT-3'; antisense, 5'-GAAAGTCCAGCTACCAAGGGAAT-3'),

human VCAM-1 (sense, 5'- GGTGGGACACAAATAAGGGTTTTGG -3'; antisense, 5'-CTTGCAATTCTTTTACAGCCTGCC-3'), human ICAM-1 (sense, 5'-GTCCCCTCAAAAGTCATCC-3'; antisense, 5'-AACCCCATTCAGCGTCACCT-3'), human β- actin (sense, 5'-CtGGAACGGTGAAGGTGACA-3'; antisense, 5'-AAGGGACTTCCTGTAACAATGCA-3'), murine VCAM-1 (sense, 5'-GTCAAAGAGGGAGACACCGT-3'; antisense, 5'-CGAGCCATCCACAGACTTTA-3'), murine E-selectin (sense, 5'-TTCGTGTaCcAATGCATCCT-3'; antisense, 5'-GGCTTCCATAGTCAGGGTGT-3'), murine ß-actin (sense, 5'-AGCGCAAGTACTCTGTGTGG- 3'; antisense, 5'-CTTGCTGAtCcaCAtCtGCT-3'), rat/murine Cezanne (sense, 5'- GGTTGGCAGCAGTTCTATCA-3'; antisense, 5'-CAAAGCTGCCCAGTTTGTTA-3') and rat ß- actin (sense, 5'-aGcGCAAGTACTCTGTGTGG-3'; antisense, 5'- CTTGCTGATCCACATCTGCT-3') using PerfeCTa SYBR Green Supermix (Quanta Biosciences) and the CFX96 Real-Time PCR Detection System (BioRad). Reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product. Data were pooled from three independent experiments and mean values were calculated with standard deviations.

Western blotting

Levels of particular proteins were measured in cytosolic or nuclear lysates prepared using the Nuclear Extraction Kit (Active Motif) by Western blotting using specific primary antibodies, HRP-conjugated secondary antibodies and chemiluminescent detection.

Immunoprecipitation of TRAF6

Cells were lysed using 30 mM Tris-HCl (pH 7.6), 120 mM NaCl, 1% Triton X-100, 2 mM KCl, 2 mM EDTA, 10% glycerol and Complete Protease Inhibitor Cocktails (Roche). Lysates were clarified by low-speed centrifugation and pre-cleared using protein-G-sepharose before immunoprecipitation using anti-TRAF6 antibodies. Beads were then washed extensively using lysis buffer. Precipitated material or lysates were analysed by Western blotting.

Animals

Male Fisher rats (F344, $RT1^{lvl}$) (170 to 210 g) were obtained commercially (Charles River, Sulzfeld, Germany). Transgenic 'gene-trapped' (GT) mice[22] in which the Cezanne gene is disrupted by removal of exons 4-7 and insertion of a splice acceptor site, LacZ open reading frame, neomycin resistance gene (Neo) and polyA tail in the third intron (B6-*Otud7btml(NCOM)Cmhd*; $Cezanne^{GT/GT}$; C57BL/6 background) were obtained from the Canadian Mouse Mutant Repository. Splicing of the LacZ-Neo-polyA sequences to the third exon of Cezanne was confirmed by RT-PCR (Online Fig. I) and is predicted to generate a fusion protein comprising the peptide MTLDMDAVLSDFVRSTGAEPGLARDLLE (encoded by exon 3 of Cezanne) linked to LacZ. Baseline phenotyping analysis carried out at the Toronto Centre for Phenogenomics as part of the North American Conditional Mouse Mutagenesis Project revealed that $Cezanne^{GT/GT}$ mice are normal in terms of gross appearance, histopathology of multiple tissues, weight gain, glucose tolerance and clinical chemistry (http://www.europhenome.org).

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Renal ischemia/reperfusion

For the induction of renal ischemia/reperfusion in rats, the abdomen was opened under inhalation anesthesia using isofluorane. The left renal artery was clamped with an atraumatic vascular clamp for 45 min. During this time the right kidney was removed. The left kidney was reperfused for 2-72 h. Renal ischemia/reperfusion in mice was carried out under inhalation anesthesia using isofluorane. In wild-type and Cezanne $^{GT/GT}$ mice, the left renal artery was clamped with an atraumatic vascular clamp for 45 min and subsequently reperfused for 6 h. Ischemia and subsequent reperfusion were confirmed at a macroscopic level by monitoring changes in tissue appearance. Principles of NIH Guide for the Care and Use of Laboratory Animals as well as the German Law on the Protection of Animals and UK Home Office regulations were followed.

Immunohistochemistry and morphological studies

Sections made from formalin-fixed, paraffin-embedded tissues were incubated in xylene for 5 min, hydrated by sequential exposure to decreasing concentrations of ethanol (100% to 50%) and water. Heat-mediated antigen retrieval was carried out in tris-sodium citrate in a standard microwave. The sections were then blocked for endogenous peroxidase activity and incubated in 20% goat serum prior to overnight incubation with primary antibodies followed by HRP-conjugated secondary antibodies and substrate (EnVision™+ Kits; Dako). Sections were then counterstained with hematoxylin and visualized by bright field microscopy. For morphological studies, sections were stained with hematoxylin and eosin prior to histological assessment. Tissue injury was assessed independently by two experienced renal researchers and classified as healthy or mild/moderate/severe injury as we described[9]. Granulocytes were identified by staining using the napthol AS-D chloroacetate esterase kit (Sigma).

Statistics

Differences between samples were analysed using an unpaired Student’s t-test or ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Hypoxia induces Cezanne via transcriptional and post-transcriptional mechanisms

Studies of cultured HUVEC revealed that Cezanne mRNA and protein levels were enhanced by exposure to 1% O_2 and remained elevated for at least 24 h following reoxygenation (Fig. 1A,B). This level of hypoxia (1% O2) also induced Cezanne in cultured microvascular EC at both the mRNA (Fig. 1C) and protein levels (Online Fig. II). By contrast, exposure of EC to 2% or 5% O_2 had little or no effect on Cezanne expression in HUVEC or HCMEC (Fig. 1D). Thus cellular responses to hypoxia were studied using 1% O2 conditions in all subsequent experiments.

We studied the molecular mechanism for Cezanne induction and focussed on the role of p38 MAP kinase and the transcription factor HIF1α, which are known to be activated by hypoxia[8,23,24]. Activation of HIF1α in response to dimethyloxallyl glycine, **desferrioxamine** or $CoCl_2$ did not influence Cezanne expression (Online Fig. III) and

silencing of HIF1α or HIF2α did not suppress Cezanne expression in hypoxic EC (Online Fig. IV), indicating that induction of Cezanne by hypoxia is HIF-independent. By contrast, pharmacological inhibition of p38 using either CT8730 or SB202190 (Fig. 1E,F) or gene silencing of p38α (Fig. 1G) suppressed the induction of Cezanne by hypoxia, indicating that p38 positively regulates Cezanne expression. We examined the mechanism of Cezanne induction by p38, which is known to influence gene expression via members of the activating transcription factor (ATF) family and also by enhancing mRNA stability[25]. We observed that ATF2 was phosphorylated in response to hypoxia in both HUVEC and microvascular EC (Fig. 2A). Thus, gene silencing studies were carried out to assess the function of ATF2 in hypoxic EC. We observed that silencing of ATF2 using two different siRNA sequences suppressed the induction of Cezanne by hypoxia (Fig. 2B). By contrast, transfection using non-targeting scrambled sequences or using sequences that target an irrelevant mRNA (SHP2) demonstrated that transfection and mRNA processing per se did not alter Cezanne induction by hypoxia (Fig. 2B). Finally, Cezanne expression was reduced by two different siRNAs designed to target Cezanne, thus validating the quantitative real-time PCR readout (Fig. 2B). Thus we conclude that ATF2 positively regulates Cezanne expression in hypoxic EC. p38 also enhanced the stability of Cezanne transcripts in hypoxic EC as pharmacological inhibition of p38 using SB202190 destabilized Cezanne mRNA in actinomycin D chase experiments (Online Fig. V). Thus we conclude that p38 induces Cezanne through both transcriptional and post-transcriptional mechanisms.

Cezanne suppresses inflammatory activation in response to hypoxia-reoxygenation by inhibiting RelA phosphorylation

We used a previously validated RNA interference method ([15] and Fig. 2B) to examine whether Cezanne regulates pro-inflammatory responses to hypoxia-reoxygenation. Silencing of Cezanne in HUVEC had no effect on basal levels of E-selectin or VCAM-1 in unstimulated cells (Fig. 3A, compare 1 & 2) but enhanced their subsequent induction by hypoxia-reoxygenation (compare 5 & 6 and 7 & 8). By contrast, silencing of Cezanne enhanced the expression of ICAM-1 in EC exposed to normoxia or hypoxia-reoxygenation (Fig. 3A, lower panel). Silencing of Cezanne using a pool of siRNA sequences from an alternative source also significantly enhanced the induction of E-selectin, VCAM-1 and ICAM-1 in EC exposed to hypoxia-reoxygenation (Online Fig. VI).

As E-selectin, VCAM-1 and ICAM-1 are induced by NF-κB, we examined whether Cezanne influences the activation of this transcription factor in response to hypoxia. Exposure of EC to hypoxia-reoxygenation enhanced Ser536 phosphorylation of RelA NF-κB sub-units but did not influence IκBα stability or nuclear localization of NF-κB (Fig. 3B). Thus hypoxia-reoxygenation activates NF-κB in EC through a non-canonical pathway that involves RelA phosphorylation. Although silencing of Cezanne led to a modest reduction in IκBα expression (Fig. 3B, upper panel), the biological significance is questionable since there was no noticeable effect of Cezanne siRNA on NF-κB nuclear localisation (Fig. 3B, lower panels). By contrast, silencing of Cezanne led to a pronounced elevation in Ser536 phosphorylation of RelA in cells exposed to hypoxia-reoxygenation (Fig. 3B, centre panel), indicating that endogenous Cezanne negatively regulates NF-κB activation and pro-inflammatory transcriptional responses under these conditions.

Cezanne prevents Lys-63 polyubiquitination of TRAF6 in response to hypoxia-reoxygenation

We examined whether Cezanne influences Lys63-polyubiquitination of TRAF6, a modification that promotes NF-κB activation in response to hypoxia[12]. Analysis of TRAF6 immunoprecipitates revealed that hypoxia-reoxygenation induced high molecular weight forms of TRAF6 that co-precipitated with Lys63 polyubiquitin in EC that were pre-treated with Cezanne-specific siRNA but not in control cultures (Fig. 4A, compare 4 and 9; Fig. 4B, compare 3 and 6). Kinetic studies demonstrated that hypoxia-reoxygenation for 4-8 h led to modification of TRAF6 in the absence of Cezanne, whereas earlier (2 h) and later time points (16-24 h) had little or no effect (Online Fig. VII). It is plausible that modification of TRAF6 requires at least 4 h exposure to hypoxia due to the requirement of de novo synthesis of a component of the ubiquitination machinery. These data indicate that endogenous Cezanne suppresses TRAF6 polyubiquitination in response to hypoxia-reoxygenation.

Cezanne reduces inflammation and injury in response to ischemia-reperfusion

Our finding that Cezanne can be induced by hypoxia *in vitro* led us to examine the expression of Cezanne in tissues exposed to ischemia *in vivo*. We observed by quantitative RT-PCR (Figs. 5A and 5C) and Western blotting (Fig. 5B) that Cezanne was induced in rat or murine kidneys exposed to ischemia-reperfusion. Immunohistochemistry was carried out to assess the cellular localization of Cezanne expression in ischemic tissues. This was performed exclusively using the murine model so that tissues from Cezanne$^{GT/GT}$ mice could be used to control for staining specificity. Immunohistochemistry using anti-Cezanne antibodies revealed staining in vascular EC, glomerular EC, podocytes and tubular epithelial cells in wild-type mice exposed to ischemia-reperfusion, whereas tissues from Cezanne$^{GT/GT}$ mice were negative (Fig. 5D). Thus we conclude that ischemia-reperfusion induces Cezanne in multiple cell types in the kidney including EC.

The influence of Cezanne on NF-κB activation, inflammation and injury was assessed by comparing responses to ischemia-reperfusion in wild-type and Cezanne$^{GT/GT}$ mice. In the first instance, we demonstrated by quantitative RT-PCR that Cezanne was expressed in several tissues in wild-type mice (highest in brain) but was absent from peripheral blood cells (Online Fig. VIII). Renal ischemia-reperfusion promoted the accumulation of granulocytes (Fig. 6A) and enhanced tissue injury (Fig. 6B and Online Fig. IX) in kidneys of wild-type mice. Of note, granulocyte accumulation and tissue injury in response to ischemia-reperfusion were significantly greater in Cezanne$^{GT/GT}$ mice compared to wild-type animals (Fig. 6 and Online Fig. IX). Similarly, expression of VCAM-1 and E-selectin (Fig. 7A) and Ser536 phosphorylation of RelA in response to ischemia-reperfusion were enhanced in Cezanne$^{GT/GT}$ mice compared to wild-type animals (Fig. 7B). Thus we conclude that ischemia induces local expression of Cezanne which protects the kidney from NF-κB-dependent inflammation and injury in response to reperfusion.

Discussion

Recent studies have shown that the assembly of polyubiquitin chains on specific signalling intermediaries promotes signalling to NF-κB in response to several stimuli including

cytokines, microbial products and hypoxia[12,26]. Our previous studies revealed that Cezanne is a deubiquitinating enzyme that suppresses NF-κB activation in response to TNFα or IL-1 by removing polyubiquitin chains from signalling intermediaries[15,18]. Here we demonstrate that Cezanne can also inhibit NF-κB-dependent inflammatory activation in response to hypoxia- reoxygenation by reducing Lys63 polyubiquitination of TRAF6. To our knowledge, Cezanne is the first example of a deubiquitinating enzyme that controls inflammatory responses to hypoxia.

Inflammatory signalling activates multiple delayed negative feedback loops that precisely control the kinetics of NF-κB and MAP kinase activation. That is, NF-κB induces molecules that feedback to inhibit signalling to NF-κB (e.g. IκBα, A20, Cezanne[15,27,29]) and/or suppress the activity of inflammatory MAP kinases (e.g. MKP-1, GADD45ß[30,31]). Here we describe a novel form of cross-talk between the NF-κB and MAP kinase pathways where p38-dependent induction of Cezanne during hypoxia suppresses NF-κB activity. Importantly, although hypoxia primed EC for NF-κB activation, it *simultaneously* induced Cezanne which blunted signalling to NF-κB during reoxygenation. Thus hypoxia-reoxygenation had minimal effects on NF-κB activation and adhesion molecule expression in unmanipulated EC but significantly enhanced inflammatory activation following silencing of Cezanne. This feed-forward inhibition pathway differs from typical delayed feedback systems because here the ‘accelerator’ (signalling to NF- κB) and ‘brake’ (Cezanne) are applied simultaneously. TNFR signalling provides a classic example of simultaneous activation of positive and negative regulators as it activates a pro- apoptotic pathway and simultaneously signals to NF-κB which induces multiple anti-apoptotic molecules[32,33]. Thus, although TNFα can prime cells for apoptosis, the execution of TNFα- mediated apoptosis usually relies on additional factors that suppress NF-κB (e.g. viral infection[34]). By analogy, although hypoxia can prime EC for inflammatory activation, the expression and/or activity of Cezanne determines whether inflammatory signalling proceeds in response to reoxygenation. Given that Cezanne can be modulated by shear stress, TNFα, IL-1 and reactive oxygen species[15,16], future studies should address whether these factors influence inflammatory responses to hypoxia-reoxygenation by altering the expression and/or activity of Cezanne.

We translated our *in vitro* findings to a murine model by demonstrating that renal inflammation and injury in response to ischemia/reperfusion is enhanced by genetic deletion of Cezanne. Although several papers have studied the function of Cezanne in cultured cells, this is the first demonstration that Cezanne protects against inflammation and injury *in vivo*. Since Cezanne was shown to be expressed in renal tissues but not in peripheral blood, we suggest that local expression of this molecule is responsible for protection. However, we cannot rule out the possibility of systemic effects entirely since a recent study revealed that Cezanne influences B cell function[17]. Immunohistochemistry revealed that Cezanne was expressed in vascular and glomerular EC in ischemic kidneys as well as epithelial cells. Thus the function of Cezanne identified in ischemic kidneys may be due, in part, to its induction in EC in response to hypoxia. The expression of Cezanne in ischemic tissues may also be related to inflammatory cytokines which are known to induce Cezanne in cultured cells[15]. Future studies using tissue-specific conditional knockout mice should now be carried out to define the specific cell populations that are regulated by Cezanne during ischemia.

Further studies are also required to examine whether Cezanne is a pleiotropic molecule that possesses divergent functions since baseline phenotyping studies revealed that Cezanne knockout mice have a reduced startle response and lower serum cholesterol levels (http://www.europhenome.org).

Our observation that endogenous Cezanne can reduce inflammation and injury in response to ischemia-reperfusion has important implications for therapeutic targeting of inflammation during transplantation of vascularised organs, bypass grafting, percutaneous coronary intervention of arteries and other procedures that involve reperfusion. Future studies should examine the potential role of Cezanne induction in ischemic preconditioning, a procedure that is known to protect tissues from subsequent inflammation and injury in response to ischemia-reperfusion[35]. In addition, strategies to enhance Cezanne expression (e.g. pharmacologically) in ischemic tissues may inform the development of novel therapies to reduce ischemia-reperfusion injury.

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Non-Standard Abbreviations

EC	endothelial cells
MAP kinase	mitogen-activated protein kinase
NF-κB	nuclear factor κB
IκB	inhibitor of κB
TRAF6	tumour necrosis factor receptor associated factor 6
IKK	IκB kinase

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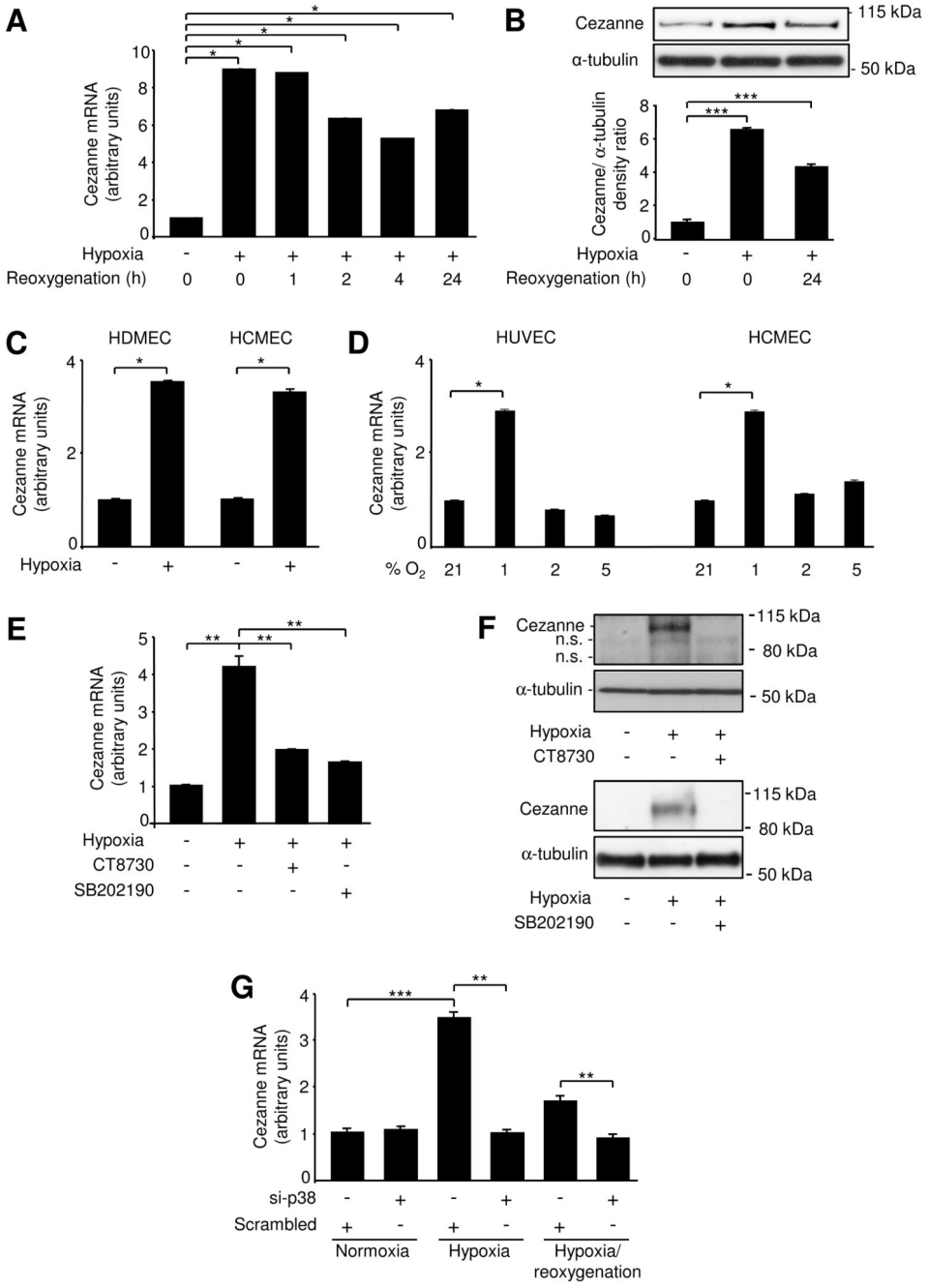

Figure 1. Hypoxia induced Cezanne via p38.

(A,B) HUVEC were exposed to hypoxia (4 h) or hypoxia followed by reoxygenation (1-24 h) or remained untreated. (A) Cezanne transcript levels were quantified by real-time PCR. Data were pooled from 3 independent experiments. (B) Cytosolic lysates were tested by Western blotting using anti-Cezanne antibodies and by using anti-α-tubulin antibodies to assess total protein levels. Representative blots (upper panels) and results from densitometry analysis of five experiments (lower panel) are shown. (C) HDMEC or HCMEC were exposed to hypoxia (4 h) or remained untreated. Cezanne transcript levels were quantified by

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real-time PCR. Data were pooled from 3 independent experiments. (D) HUVEC or HCMEC were exposed to 1%, 2%, 5% or normoxia (21%) for 4 h. Cezanne transcript levels were quantified by real-time PCR. Data were pooled from 3 independent experiments. (E,F) HUVEC were exposed to CT8730 (1 μM) or SB202190 (50 μM) for 1 h or were treated with vehicle alone and then exposed to hypoxia (4 h) or remained untreated. (E) Cezanne transcript levels were quantified by real-time PCR. (F) Cytosolic lysates were tested by Western blotting using anti-Cezanne antibodies and by using anti-α-tubulin antibodies to assess total protein levels. n.s., non-specific band. (G) HUVEC were treated with p38α-specific siRNA (si-p38) or with a scrambled, non-targeting sequence and were then exposed to hypoxia (4 h) or hypoxia (4 h) followed by reoxygenation (4 h) or remained untreated (normoxia). Cezanne transcript levels were quantified by real-time PCR.

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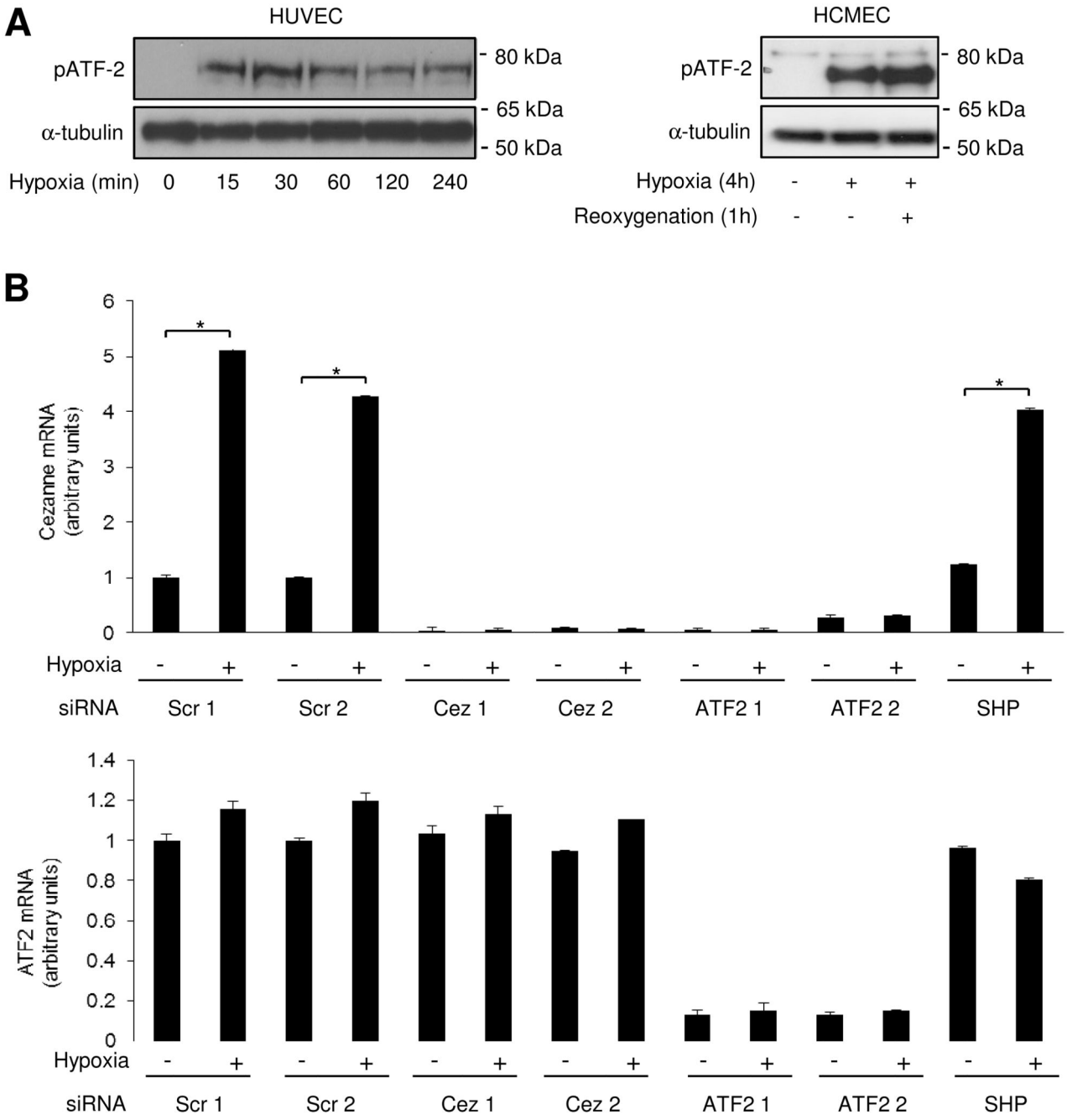

Figure 2. Hypoxia induced Cezanne via ATF2.

(A) HUVEC or HCMEC were exposed to hypoxia for 15 min-4 h or remained untreated (normoxia). Some cultures were then reoxygenated for 1 h. Cytosolic lysates were tested by Western blotting using anti-phosphorylated ATF2 antibodies or by using anti-α-tubulin antibodies to assess total protein levels. Data are representative of three independent experiments. (B) HUVEC were treated with siRNA sequences that target Cezanne (Cez 1, Dharmacon; Cez 2, SMARTpool), ATF2 (ATF2 1, Dharmacon; ATF2 2, SMARTpool) or SHP2, or with scrambled, non-targeting sequences as a control (Scr). They were then

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exposed to hypoxia (4 h) or remained untreated (normoxia). Levels of Cezanne or ATF2 were quantified by real-time PCR. Data were pooled from 3 independent experiments.

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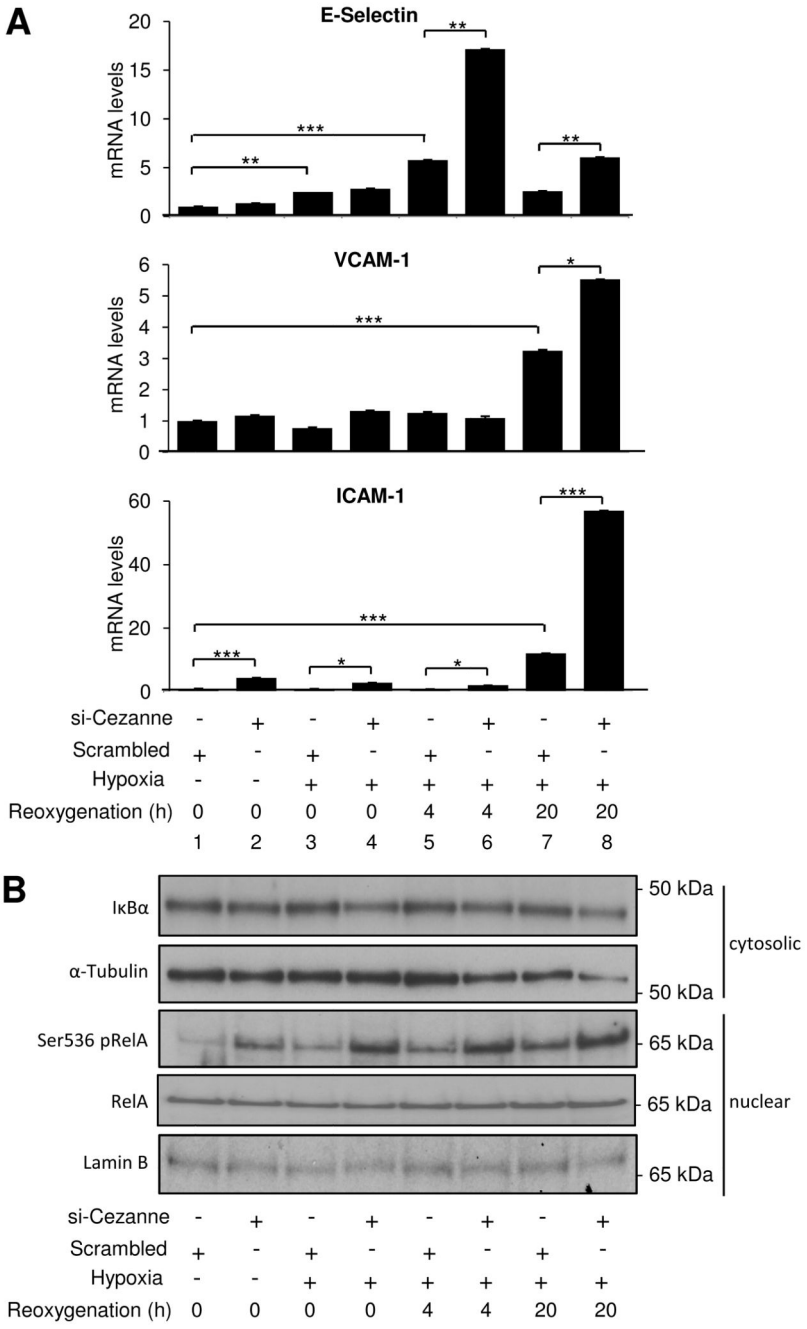

Figure 3. Cezanne suppressed inflammatory activation in response to hypoxia-reoxygenation.

HUVEC were treated with Cezanne-specific siRNA (si-Cezanne from Dharmacon) or with a scrambled, non-targeting sequence and were then exposed to hypoxia (4 h) or hypoxia (4 h) followed by reoxygenation (4-20 h) or remained untreated. (A) Levels of E-selectin, VCAM- 1 or ICAM-1 transcripts were quantified by real-time PCR. Data were pooled from three independent experiments. (B) Cytosolic or nuclear lysates were tested by Western blotting using anti-IκBα, anti-RelA or anti-Ser536 phosphorylated RelA antibodies and by

using anti-α-tubulin or anti-Lamin B antibodies to assess total protein levels. Data are representative of three independent experiments.

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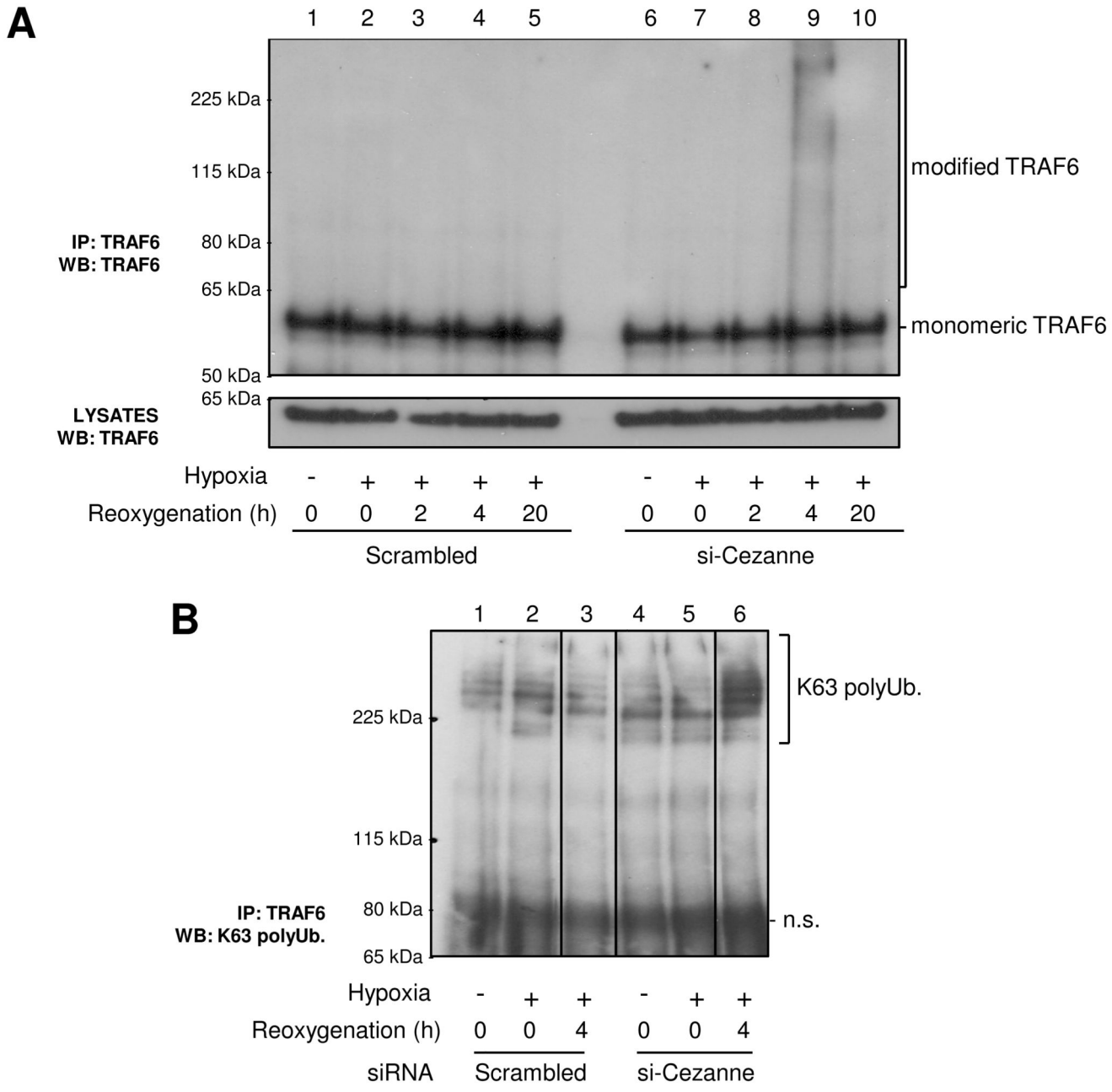

Figure 4. Cezanne suppressed TRAF6 polyubiquitination in response to hypoxia-reoxygenation.

HUVEC were treated with Cezanne-specific siRNA (si-Cezanne from Dharmacon) or with a scrambled, non-targeting sequence and were then exposed to hypoxia (4 h) or hypoxia (4 h) followed by reoxygenation (2-20 h) or remained untreated. (A) TRAF6 immunoprecipitates or lysates were tested by Western blotting using anti-TRAF6 antibodies. (B) TRAF6 immunoprecipitates were tested by Western blotting using antibodies that recognise Lys63-polyubiquitin. n.s., non-specific. Data are representative of three independent experiments that gave closely similar results.

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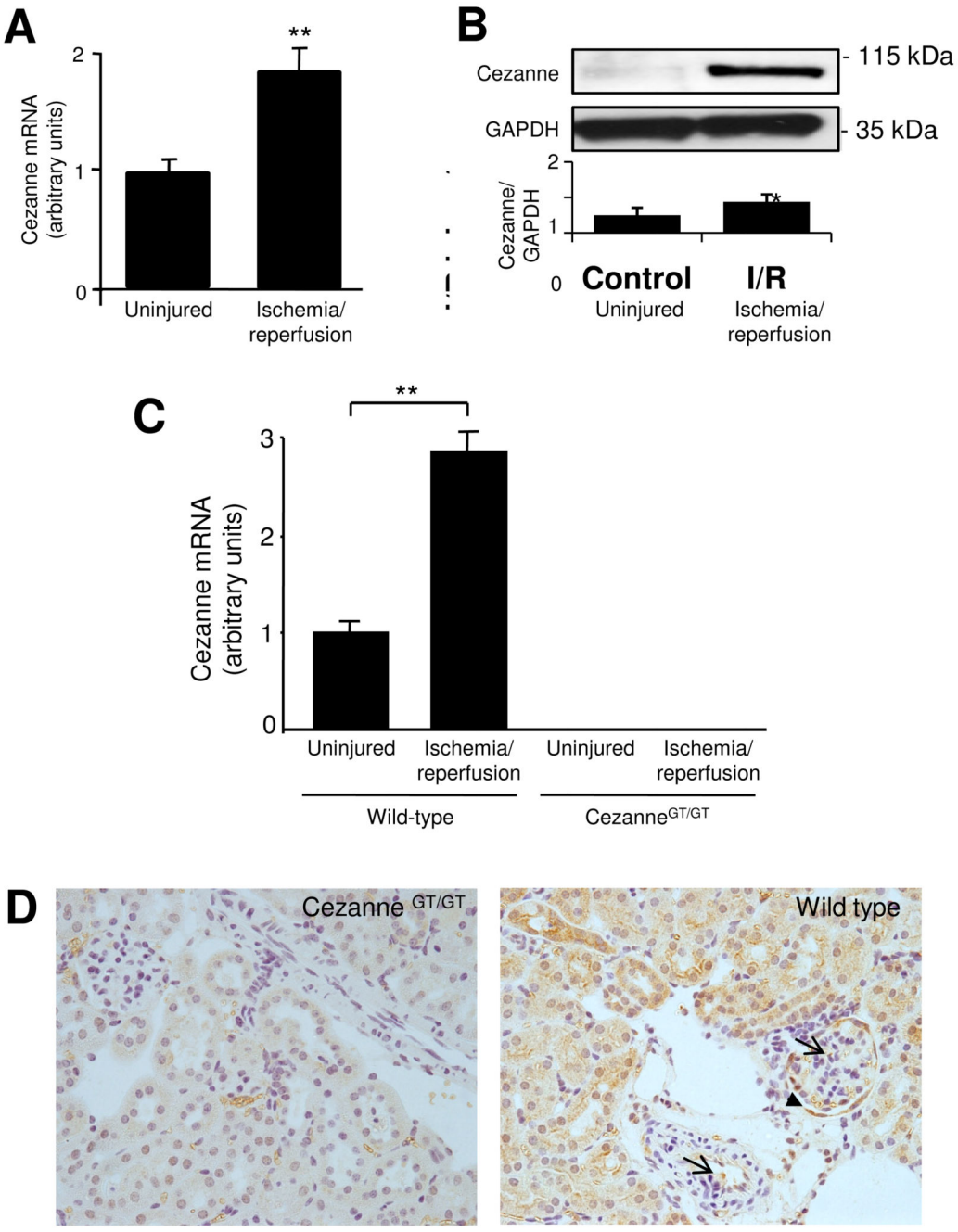

Figure 5. Cezanne was induced by renal ischemia *in vivo*.

(A,B) Expression levels of Cezanne were assessed in left kidneys of male Fisher rats (n=4) that were exposed to ischemia followed by reperfusion. Basal levels of Cezanne were measured in the uninjured right kidney of each animal. (A) Cezanne transcript levels were quantified in kidneys exposed to ischemia-reperfusion (6 h) and uninjured kidneys by real-time PCR and normalised by quantifying β-actin transcripts. (B) Lysates from kidneys exposed to ischemia-reperfusion (6 h) or uninjured kidneys were tested by Western blotting using anti-Cezanne antibodies or by using anti-α-tubulin antibodies to assess total protein

levels. A representative blot and results from densitometry analysis are shown. (C,D) Left kidneys of wild-type or Cezanne$^{GT/GT}$ mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 h whereas contralateral kidneys were uninjured. (C) Cezanne transcript levels were quantified in kidneys exposed to ischemia-reperfusion and uninjured kidneys by real-time PCR and normalised by quantifying β-actin transcripts. (D) Cezanne expression was assessed by immunohistochemistry in kidneys exposed to ischemia-reperfusion. Positive staining was observed in wild-type tissues in vascular and glomerular EC (arrows), in podocytes (arrowhead) and epithelial cells.

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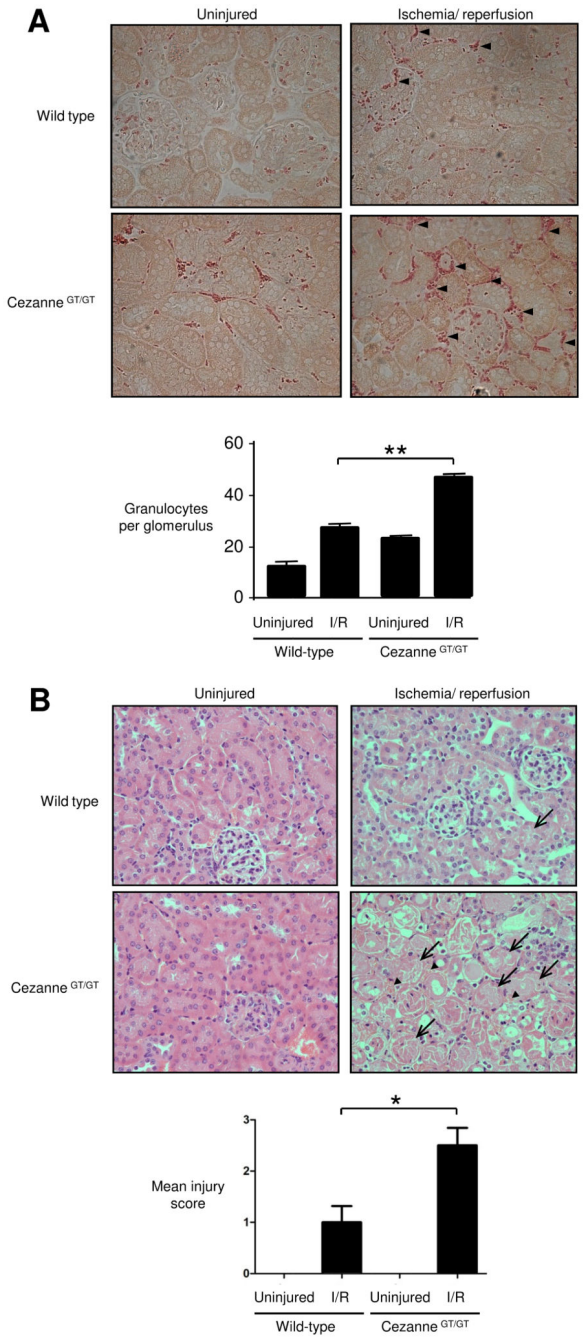

Figure 6. Cezanne reduced inflammation and injury in response to ischemia-reperfusion.

Left kidneys of wild-type or Cezanne$^{GT/GT}$ mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 h whereas contralateral kidneys were uninjured. (A) Granulocytes were identified by napthol AS-D chloroacetate esterase staining. Representative images are shown and positive cells are indicated (arrow heads) (upper panels). Granulocytes were counted manually and data were pooled from multiple animals per group (lower panels). (B) Tissue sections were stained with hematoxylin and eosin prior to histological assessment. Representative images are shown with areas of acute tubular

necrosis (arrows) and haemorrhage (arrow heads) indicated (upper panels). Quantitative assessment of tissue injury was carried out by two experienced renal researchers and average scores are shown (lower panels). 0, healthy; 1, mild injury; 2, moderate injury; 3, severe injury.

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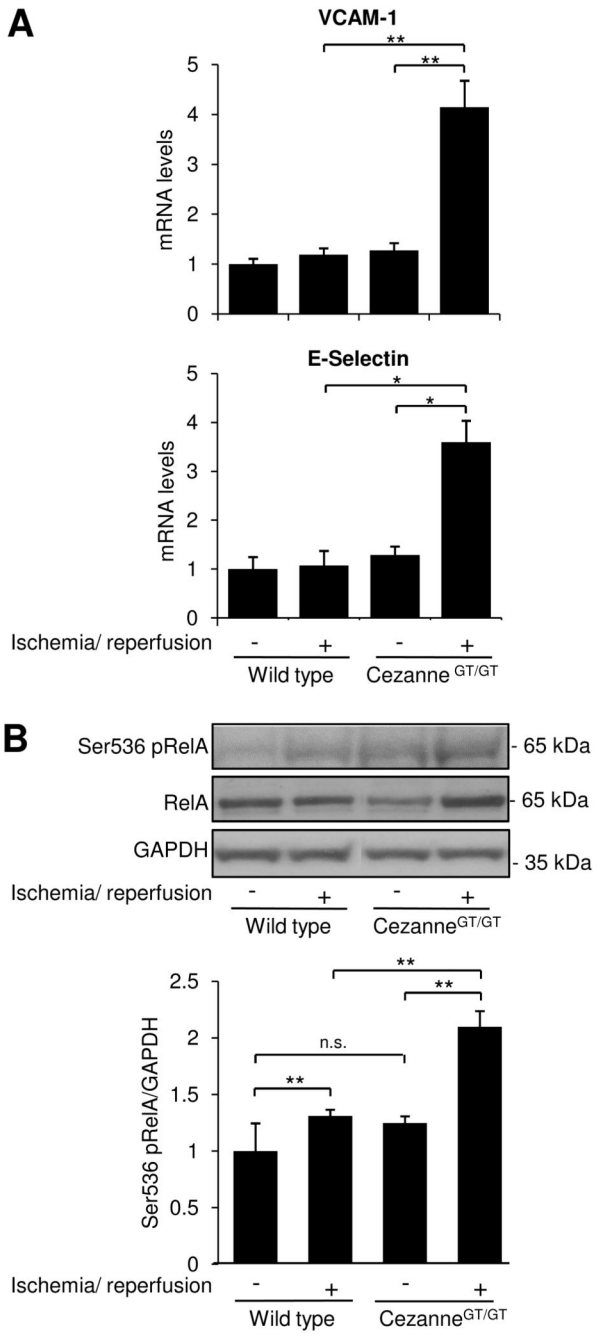

Figure 7. Cezanne suppressed NF-κB activation and adhesion molecule expression in response to ischemia-reperfusion.

Left kidneys of wild-type or Cezanne$^{GT/GT}$ mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 h whereas contralateral kidneys were uninjured. (A) Levels of VCAM-1 or E-selectin transcripts were quantified by real-time PCR. (B) Lysates were tested by Western blotting using anti-RelA or anti-Ser536 phosphorylated antibodies or by using anti-GAPDH antibodies to assess total protein levels. Representative blots and results from densitometry analysis are shown.