BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 171–180

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

The melanocortin MC_1 receptor agonist BMS-470539 inhibits leucocyte trafficking in the inflamed vasculature

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Background and purpose: Over three decades of research evaluating the biology of melanocortin (MC) hormones and synthetic peptides, activation of the MC type 1 (MC_1) receptor has been identified as a viable target for the development of novel anti-inflammatory therapeutic agents. Here, we have tested a recently described selective agonist of MC_1 receptors, BMS-470539, on leucocyte/post-capillary venule interactions in murine microvascular beds.
Experimental approach: Intravital microscopy of two murine microcirculations were utilized, applying two distinct modes of promoting inflammation. The specificity of the effects of BMS-470539 was assessed using mice bearing mutant inactive MC_1 receptors (the recessive yellow e/e colony).
Key results: BMS-470539, given before an ischaemia–reperfusion protocol, inhibited cell adhesion and emigration with no effect on cell rolling, as assessed 90 min into the reperfusion phase. These properties were paralleled by inhibition of tissue expression of both CXCL1 and CCL2. Confocal investigations of inflamed post-capillary venules revealed immunostaining for MC_1 receptors on adherent and emigrated leucocytes. Congruently, the anti-inflammatory properties of BMS-470539 were lost in mesenteries of mice bearing the inactive mutant MC_1 receptors. Therapeutic administration of BMS-470539 stopped cell emigration, but did not affect cell adhesion in the cremasteric microcirculation inflamed by superfusion with platelet-activating factor.
Conclusions and implications: Activation of MC_1 receptors inhibited leucocyte adhesion and emigration. Development of new chemical entities directed at MC_1 receptors could be a viable approach in the development of novel anti-inflammatory therapeutic agents with potential application to post-ischaemic conditions.
British Journal of Pharmacology (2010) **160,** 171–180; doi:10.1111/j.1476-5381.2010.00688.x; published online 19 March 2010

Keywords: neutrophil trafficking; endogenous anti-inflammation; melanocortins; new chemical entities; microcirculation; endothelium activation; tissue chemokines; anti-inflammatory GPCR

Abbreviations: ACTH, adrenocorticotrophin; αMSH, alpha-melanocyte-stimulating hormone; IR, ischaemia–reperfusion; LPS, lipolysaccharide; PAF, platelet-activating factor

Introduction

The concept of active resolution in inflammation has, in recent years, gained such momentum that several studies are detailing the mechanisms that ensure the correct time – and spatial – dependence of this important phase of the host response. Specific pathways are activated in the body to ensure that, for instance, the process of leucocyte migration, which is incited by several classes of pro-inflammatory mediators and adhesion molecules (Ley *et al.*, 2007), will subside over time in an active mode (Serhan and Savill, 2005; Gonzalez-Rey and Delgado, 2007; Serhan *et al.*, 2007).

α-Melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophin (ACTH) are endogenous polypeptides that belong to the group of endogenous anti-inflammatory mediators (Gonzalez-Rey and Delgado, 2007; Brzoska *et al.*, 2008). These molecules activate specific receptors, melanocortin (MC) receptors (nomenclature follows Alexander *et al.*, 2009), which inhibit, on one hand, the production of pro-inflammatory cytokines from target cells (Catania *et al.*, 2004), and, on the other, put in motion pro-resolving processes including the induction of haem oxygenase 1 (Lam

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Received 26 October 2009; revised 4 January 2010; accepted 14 January 2010

et al., 2005). In integrated systems, these molecular and cellular events would lead to a tight control on the experimental inflammatory response preventing its overshooting.

From a pathophysiological perspective, compelling evidence for the inhibitory functions of this pathway derives from the exacerbation of colitis observed in mice bearing an inactive MC_1 receptor (the recessive yellow e/e mouse colony) (Maaser *et al*., 2006). On the other hand, MC_3 receptor null mice present a higher degree of vascular inflammation following an ischaemia–reperfusion (IR) insult (Leoni *et al*., 2008). This response is associated with higher levels of pro-inflammatory cytokines as measured in injured tissues. The same holds true in MC_3 null mice after 3 months of high-fat diet, where augmented tissue expression of pro-inflammatory chemokines occurs during development of an obese-like status (Trevaskis *et al*., 2007).

Natural and synthetic MC peptides bring about homeostatic and anti-inflammatory actions by activating either MC_1 or MC_3 receptors. Utilization of agonists with different degrees of selectivity for these receptors produces remarkable tissue-protective and anti-inflammatory effects (Brzoska *et al*., 2008). Three decades of research into the biology of MC peptides and their receptors on their effect on innate immunity presents an opportunity to exploit this immunomodulatory system for therapeutic development (Catania *et al*., 2004; Getting, 2006; Brzoska *et al*., 2008). Modification of short sequences of natural MCs is a viable way forward in drug development (Grieco *et al*., 2000; Brzoska *et al*., 2008; Doi *et al*., 2008). Another way forward would lie in the identification and development of novel chemical entities that would activate, selectively, either MC_1 or MC_3 receptors, and this goal has recently been achieved for MC_1 receptors. The compound, BMS-470539, binds to human MC_1 receptors in the low nanomolar range, and, in mice, inhibits lipopolysaccharide (LPS)-induced systemic tumour necrosis factor (TNF) release and LPS-induced leucocyte migration into the lung (Kang *et al*., 2006).

Our recent study performed with MC_3 receptor null mice demonstrated expression of MC_1 receptor mRNA and protein in the post-ischaemic tissue (Leoni *et al*., 2008); we tested here whether BMS-470539 would exert anti-inflammatory actions on the vascular inflammation in the mesentery that follows an IR procedure. These data have been extended to another protocol for intravital microscopy using a distinct microvascular bed (cremasteric microcirculation) and inflammatory stimulus [topically applied platelet-activating factor (PAF)]. Finally, we determined whether these pharmacological effects of BMS-470539 were mediated by activation of endogenous MC_1 receptors using mice bearing a mutant and inactive receptor.

Materials and methods

Animals

All animal care and experimental protocols complied with the guidelines laid down by the Ethical Committee for the Use of Animals, Barts and The London School of Medicine and the Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Male mice (2–3 weeks old, ~20 g body weight) were maintained on a standard chow pellet diet and had free access to water, with a 12 h light/dark cycles. Wild-type (WT) animals (strain C57BL/6J; B & K, Hull, UK) were used 7 days after arrival. The MC_1 receptor recessive yellow (e/e) mouse colony bearing a frameshift mutation in the MC_1 receptor gene (Robbins *et al*., 1993) were originally a gift from Dr Nancy Levin (Trega Bioscience, San Diego, CA, USA).

In vivo *models of vascular inflammation*

Intravital microscopy in the mesenteric microcirculation. Intravital microscopy was performed as previously reported (Leoni *et al*., 2008). The mice were anaesthetized with a mixture of xylazine (7.5 $mg \cdot kg^{-1}$) and ketamine (150 $mg \cdot kg^{-1}$), and kept warm at 37°C with a heating pad. A polyethlylene catheter (PE-10 with an internal diameter of 0.28 mm) was placed into the internal jugular vein for administration of drugs. Mesenteric ischaemia was induced with a micro-aneurysm clip (Harvard Apparatus, Kent, UK), clamping the superior mesenteric artery for 35 min. The clip was then removed, and reperfusion was allowed for 90 min (for evaluation of white blood cell reactivity). Sham-operated animals underwent the same surgical procedure except clamping of the superior mesenteric artery.

The mesenteric vascular bed was exteriorized; after positioning the microcirculation under the microscope, a 5 min equilibration period preceded the recording of quantitative measurements. Analyses of leucocyte–endothelium interactions were made in three to four randomly selected post-capillary venules (diameter between 20 and 40 μm; visible length of at least 100 μm) for each mouse.

Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. White blood cell rolling velocity (V_{WBC}) was determined from the time required for a leucocyte to roll a given distance along the length of the venule, and is reported in $\mu m \cdot s^{-1}$. Rolling cell flux was determined by counting the number of leucocytes passing a reference point in the venule per minute, and expressed as cells per minute ($cell \cdot min^{-1}$). Leucocyte adhesion was measured by counting clearly visible cells on the vessel wall in a 100 μm stretch. An adherent cell was defined as a cell that had remained stationary for 30 s or longer. Leucocyte emigration from the microcirculation into the tissue was calculated by counting the number of cells in a $100 \times 50\ \mu m^2$ area on both sides of the 100 μm vessel segment. Red blood cell centreline velocity was measured in venules with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, Dallas, TX, USA), and venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8000 [(red blood cell velocity/1.6)/venular diameter].

Drug treatment. The selective MC_1 receptor agonist BMS-470539 (Herpin *et al*., 2003) was used both in WT and MC_1 receptor recessive e/e mice. The compound (MW = 559.697) was given in a dose range and route of administration, shown to be inhibitory in three distinct models of inflammation (Kang *et al*., 2006). Therefore, doses of 18.47, 6.16 and 2.05 $mg \cdot kg^{-1}$ (corresponding to 33, 11 and 2.9 $\mu mol \cdot kg^{-1}$,

respectively) were given i.v. (via jugular vein) in a fresh solution of PBS (100 μL per mouse) before inducing ischaemia.

Intravital microscopy in the cremasteric microcirculation. Intravital microscopy was used to observe PAF-induced leucocyte responses within the cremasteric microcirculation, adopting a protocol used to monitor events in the microcirculation in real time (Chatterjee *et al.*, 2005). Briefly, the cremaster was dissected free of skin and fascia, opened and superfused with bicarbonate-buffered saline (in mM: 131.92, NaCl; 3.35, KCl; 1.16, $MgSO_4$; 17.97, $NaHCO_3$; and 1.98, $CaCl_2$, pH 7.4, 37°C) at a rate of 2 mL·min^{-1}. During the 30 min stabilization period, a post-capillary venule (diameter between 20 and 40 μm; length > 100 μm) was selected; then, 100 nM PAF (C16 form: $C_{26}H_{54}NO_7P$; Sigma-Aldrich, Poole, UK) was added to the superfusion buffer. One minute recordings were made with a Hamamatsu C9300 digital camera (Intelligent Imaging Innovations, Göttingen, Germany) every 15 min up to 120 min. In some experiments, 60 min after PAF stimulation, BMS-470539 was administered i.v. at dose 33 μmol·kg^{-1}. Leucocyte cell rolling, firm adhesion and transmigration in post-capillary venules with a wall shear rate ≥ 500·s^{-1} were quantified as previously described.

Ex-vivo *analyses*

ELISA measurements. At the end of the intravital microscopy procedure, mesentery tissues were harvested and stored at –80°C. Mesenteric tissue fragments of sham-operated animals and mice subjected to IR were homogenized in 1 mL of PBS containing anti-proteases (0.1 mM phenylmethyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 IU aprotinin A) and 0.05% Tween 20. Quantitative ELISA to monitor tissue content of mouse CCL2 (MCP-1) and CXCL1 (KC) was run according to the manufacturer's instructions (R&D System Europe, Oxford, UK).

Tissue myeloperoxidase (MPO) activity. Leucocyte MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) following a well-validated protocol (Cuzzocrea *et al.*, 1997; Gavins *et al.*, 2005; Leoni *et al.*, 2008). Briefly, mesenteric tissue samples from sham- and I/R-treated mice were homogenized in PBS containing 0.5% hexadecyl trimethylammonium bromide (HTAB) detergent. The homogenate was centrifuged at 13 000× *g* for 5 min prior to adding 20 μL supernatant volumes to 160 μL of 2.8 mM of TMB, and 20 μL of 0.1 mM of H_2O_2 in 96-well plates. The plates were incubated for 5 min at room temperature, and optical density was read at 620 nm using GENios (TECAN, Reading, UK). Assays were performed in duplicate and normalized for protein content (BCA protein assay, Pierce, UK).

Confocal analyses. Whole-mount immunostaining of tissues was performed as previously described with few modifications (Voisin *et al.*, 2010). Briefly, the mice were humanely killed, and tissues of interest (cremaster muscle and mesenteric tissue) were dissected and immediately fixed by placing into PBS plus 4% paraformaldehyde for 30 min at 4°C. Following fixation, whole-mounted tissues were blocked and permeabilized in PBS containing 10% normal goat serum, 10% FCS, 5% normal mouse serum and 0.5% Triton X-100 for 2 h at room temperature. The tissues were then immunostained with antibodies against the leucocyte marker CD45 (APC-conjugated anti-CD45, Cambridge Bioscience, Cambridge, UK), the α-smooth muscle actin (α-SMA) to detect the pericytes, and thus the vasculature (cy3-conjugated anti-α-SMA, Sigma-Aldrich) and the anti-MC_1 receptor (Sigma) in PBS + 10% FCS at 4°C for 3 days. Following three washes in PBS, the tissues were incubated with a 488-conjugated anti-rabbit secondary antibody, for 3–4 h at 4°C in PBS + 10% FCS. The samples were then viewed using a Leica SP5 confocal (Leica Microsystems, Milton Keynes, UK) incorporating a ×20 water-dipping objective (NA: 1.0) at 20–24°C. Z-stack images acquired with sequential scanning of the different channels were used for 3D reconstruction of whole vessels (200 μm length; four to six vessels per tissue) with the image-processing software IMARIS (Bitplane, Zurich, Switzerland).

Data analysis

All data are reported as mean ± SEM of *n* observations, using at least five mice per group. Statistical evaluation was performed using ANOVA (Prism GraphPad software) with Bonferroni test for *post hoc* analyses, taking a P value < 0.05 as significant.

Materials

Ketamine hydrochloride was from Hoffman-La Roche, Basel Switzerland, and xylazine was from Janssen Pharmaceutica, Beerse, Belgium. The components of the MPO assay [TMB, H_20_2 (30%), HTAB and MPO from human leucocytes] were all from Sigma Aldrich. BMS-470539 was a generous gift from Dr Timothy Herpin (Bristol-Meyers Squibb).

Results

Effects of BMS-470539 on the mesenteric microcirculation

Application of the 35 + 90 min IR procedure to the mouse mesentery elicited the expected high degree of vascular inflammation in post-capillary venules (Figure 1). A sharp reduction in rolling velocity, associated with ~3-fold increase in cell adhesion and emigration, could be consistently measured in IR tissues compared to sham-operated tissues.

Treatment of mice with BMS-470539 did not modify the IR-induced reduction in V_{WBC} of rolling leucocytes (Figure 1A). In contrast, a dose-dependent inhibition of the extent of IR-induced leucocyte adhesion (Figure 1B) and emigration (Figure 1C) was observed, with significant reduction at doses of 6.16 and 18.47 mg·kg^{-1}. The top dose of BMS-470539 (18.47 mg·kg^{-1}; 660 nmol per mouse) brought IR-induced values of cell adhesion and emigration back to those measured in sham-operated mice, whereas the intermediate dose of 6.16 mg·kg^{-1} (corresponding to 123 nmol per mouse) significantly and selectively affected cell adhesion (~50% reduction; P < 0.05) (Figure 1B). The lowest dose tested of 2.05 mg·kg^{-1} was ineffective on all parameters under observation.

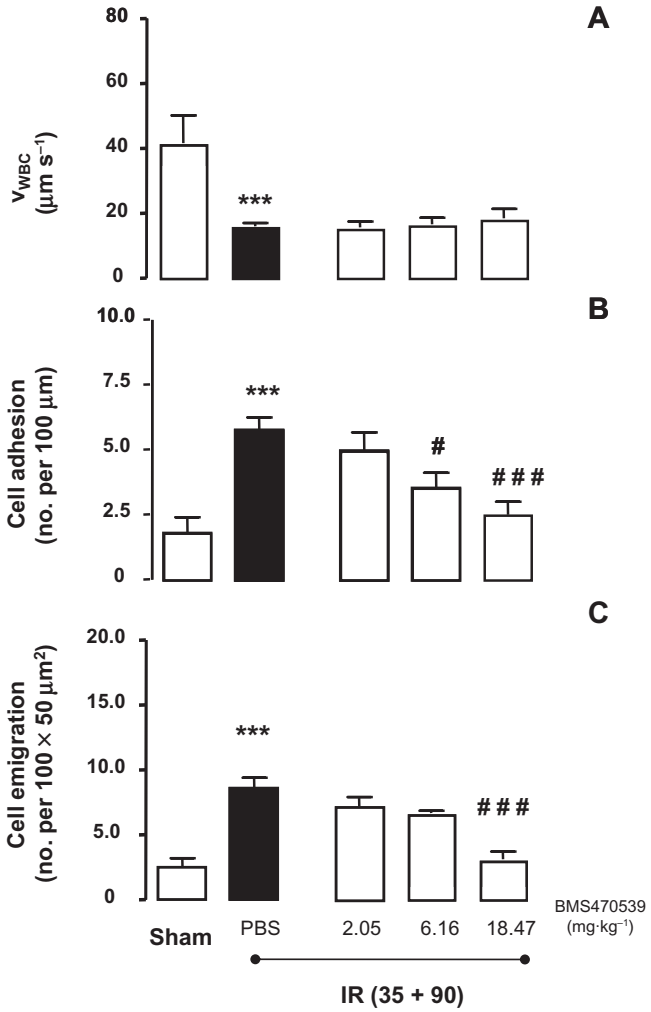

Figure 1 Anti-inflammatory properties of BMS-470539 in the post-ischaemic mesenteric microcirculation. The mice were subjected to occlusion of the superior mesenteric artery (35 min) followed by 90 min reperfusion. A sham group (laparotomy but no occlusion of the artery) was also analysed. Vehicle or BMS-470539 was given i.v. (prior to inducing ischaemia). Cellular responses in the inflamed post-capillary venule were determined 90 min post-reperfusion, monitoring cell rolling as V_{WBC} (A), cell adhesion (B) and cell emigration (C). Data are mean ± SEM of six mice per group. ***$P < 0.001$, PBS I/R versus respective sham group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus respective vehicle I/R group.

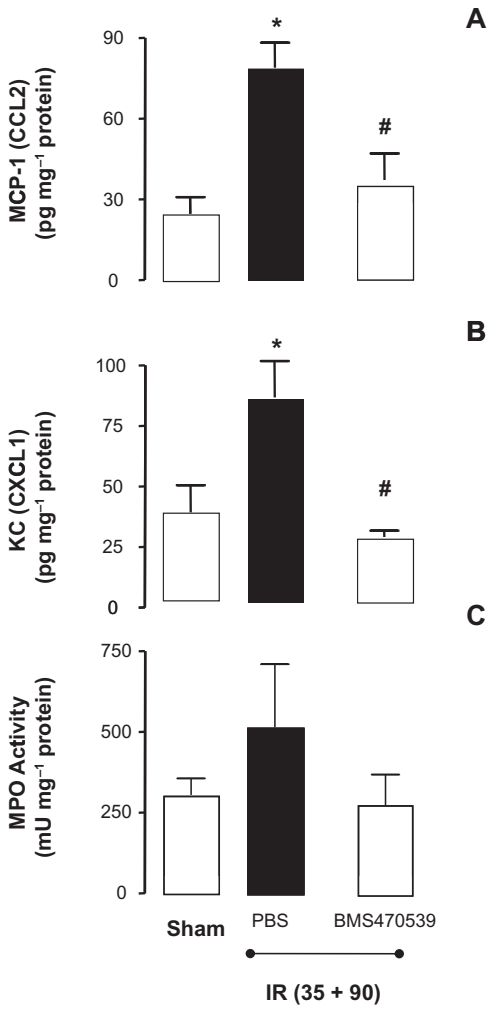

Figure 2 BMS-470539 reduces IR-induced tissue chemokine expression. WT mice were treated as in Figure 1, with BMS-470539 being given at the highest dose of 18.57 mg·kg^{-1}. A sham group (laparotomy but no occlusion of the artery) was also analysed. At the end of the IR protocol, mesenteries were homogenized and protein extracts used for the ELISA assays to determine tissue content of CCL2 (A), CXCL1 (B) and MPO activity (C). Data are mean ± SEM of six mice per group. *$P < 0.05$ versus respective sham group; #$P < 0.05$ versus respective vehicle I/R group.

These inhibitory properties displayed by BMS-470539 prompted us to determine other parameters of mesenteric tissue inflammation, focusing on the most effective dose of 18.47 mg·kg^{-1}. Figure 2 reports these data displaying the reduction in tissue levels of CCL2 (Figure 2A) and CXCL1 (Figure 2B) upon BMS-470539 treatment. For both chemokines, tissue expression of these chemokines was reduced to levels similar to those measured in sham-operated tissue samples (Figure 2A,B). MPO activity was increased after the IR procedure, and although not statistically significant, treatment with BMS-470539 resulted in a trend towards normalization of MPO activity (Figure 2C).

In recessive yellow e/e mice, the IR procedure produced a high degree of vascular inflammation, with marked attenuation in V_{WBC} and increments in the extent of cell adhesion and emigration (Figure 3). When statistical comparisons were made for each variable, no significant difference between WT and recessive yellow e/e mice emerged for either cell rolling, adhesion or emigration (compare Figure 3 with Figure 1).

Treatment of recessive yellow e/e mice with BMS-470539, given i.v. at the top dose of 18.47 mg·kg^{-1}, did not affect any of the IR-induced vascular responses, with no effect on V_{WBC} (Figure 3A), cell adhesion (Figure 3B) or cell emigration (Figure 3C).

To analyse which cell could be the target of the MC receptor inhibition, immunostaining of whole-mount mesentery subjected to IR injury was performed and viewed by confocal microscopy. Specifically, following inflammation, tissues were collected, fixed and immunostained for the pan-leucocyte marker CD45 and α-SMA to detect the pericytes of the venular wall, together with an anti-MC_1 receptor or isotype control

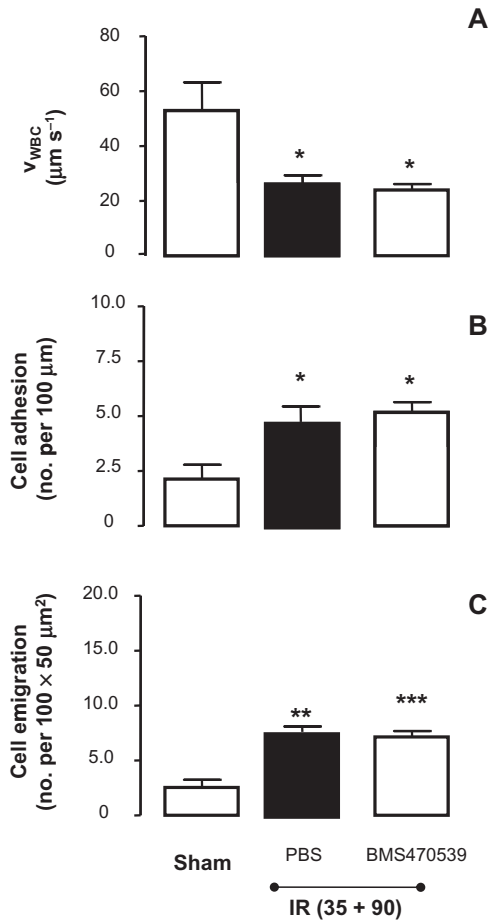

Figure 3 Lack of effect of BMS-470539 in the recessive yellow e/e mouse mesentery. Recessive yellow e/e (bearing an inactive mutant MC_1 receptor) mice were subjected to occlusion of the superior mesenteric artery for 35 min, followed by reperfusion. A sham group (laparotomy but no occlusion of the artery) was also analysed. Cellular responses in the inflamed post-capillary venule were determined 90 min post-reperfusion, monitoring cell rolling as V_{WBC} (A), cell adhesion (B) and emigration (C). Data are mean ± SEM of six mice per group. $^*P < 0.05$, $^{**}P < 0.01$ versus respective sham group.

antibody. The 3D reconstructed images of the inflamed tissues showed specific immuno-staining of transmigrated leucocytes for MC_1 receptors as compared with isotype control treated tissues (Figure 4A). Of note, MC_1 receptors were highly expressed inside, but also, at a lower extent on the surface of the transmigrated and adherent leucocytes (Figure 4B,C). The immunostaining of the tissues also demonstrated specific staining for MC_1 receptors on the luminal side of the venular wall (Figure 4C).

The pharmacological effects produced by administration of compound BMS-470539 on the inflamed vasculature were not secondary to changes in the haemodynamic parameters of the vessels under investigation. This is shown in Table 1, where values of cell flux and wall shear rate were modified by 35 + 90 min IR, but not further modified by application of BMS-470539.

Effects of BMS-470539 on the cremasteric microcirculation

To further elucidate the pharmacological potential of BMS-470539, as well as to determine the pathophysiological relevance of endogenous MC_1 receptors, the next set of experiments was conducted within a different vascular bed, the mouse cremaster muscle microcirculation. The cremaster is a skeletal muscle widely used to study mechanisms of white blood cell interactions with the post-capillary venules (Dangerfield *et al.*, 2002; Young *et al.*, 2004). It offers a higher degree of stability and so it is suitable to be inflamed over time, allowing the temporal monitoring of the cascade of events within the vasculature that characterizes the early phase of inflammation.

Firstly, we determined expression of MC_1 receptors in vascular cells also when these experimental conditions were applied. Supporting Information Figure S1 shows the MC_1 receptor immunoreactivity detected in the inflamed vessels, with a marked signal deriving from intravascular (adherent), as well as extravasated leucocytes (Supporting Information Figure S1B). Co-staining with CD45, a pan-leucocyte marker, validated this observation, so that a high (>95%) degree of co-localization between CD45 and MC_1 positivity was evident (Supporting Information Figure S1D). Controls for the immunoreaction and subsequent confocal analyses are shown in Supporting Information Figure S1A,C.

PAF superfusion of the cremasteric microcirculation produced a time-dependent inflammatory response with reduction in V_{WBC} (not shown), and a time-dependent increase in cell adhesion (Figure 5A) and emigration (Figure 5B). At 60 min post-PAF superfusion, the extent of cell adhesion and emigration was approximately 50–70% of the response measured at the 2 h time-point (Figure 5). At this juncture, the mice were split into two groups, receiving either an intravenous bolus of vehicle or of BMS-470539. Treatment with this MC_1 receptor agonist, using this therapeutic protocol, did not affect cell adhesion (Figure 5A), but produced a marked blockade of cell emigration (Figure 5B). Table 2 reports haemodynamic parameters in the cremaster.

In line with the results obtained with the mesentery protocol, there was no difference between WT and the recessive yellow e/e mouse with respect to the cellular responses promoted by PAF (Supporting Information Figure S2). Importantly, BMS-470539 was ineffective in altering the degree of cell emigration when administered to recessive yellow e/e mice (Supporting Information Figure S3) supporting, again, the active involvement of MC_1 receptors in transducing the *in vivo* anti-inflammatory properties of this compound.

Discussion

In this study, we have determined the inhibitory properties of a selective MC_1 receptor agonist, BMS-470539, on the early phases of the inflammatory response, namely the interaction between circulating white blood cells and post-capillary venules. The data produced indicate that activation of mouse MC_1 receptors by BMS-470539 exerts potent inhibition on the processes of cell adhesion and, in particular, emigration. Of interest, a significant expression of MC_1 receptor expression in recruited leucocytes was noted; however, this receptor did not seem to play a pathophysiological role *per se* in these experimental conditions.

The interest in MCs has grown over the years, so that three decades of research into the biology of these natural – and

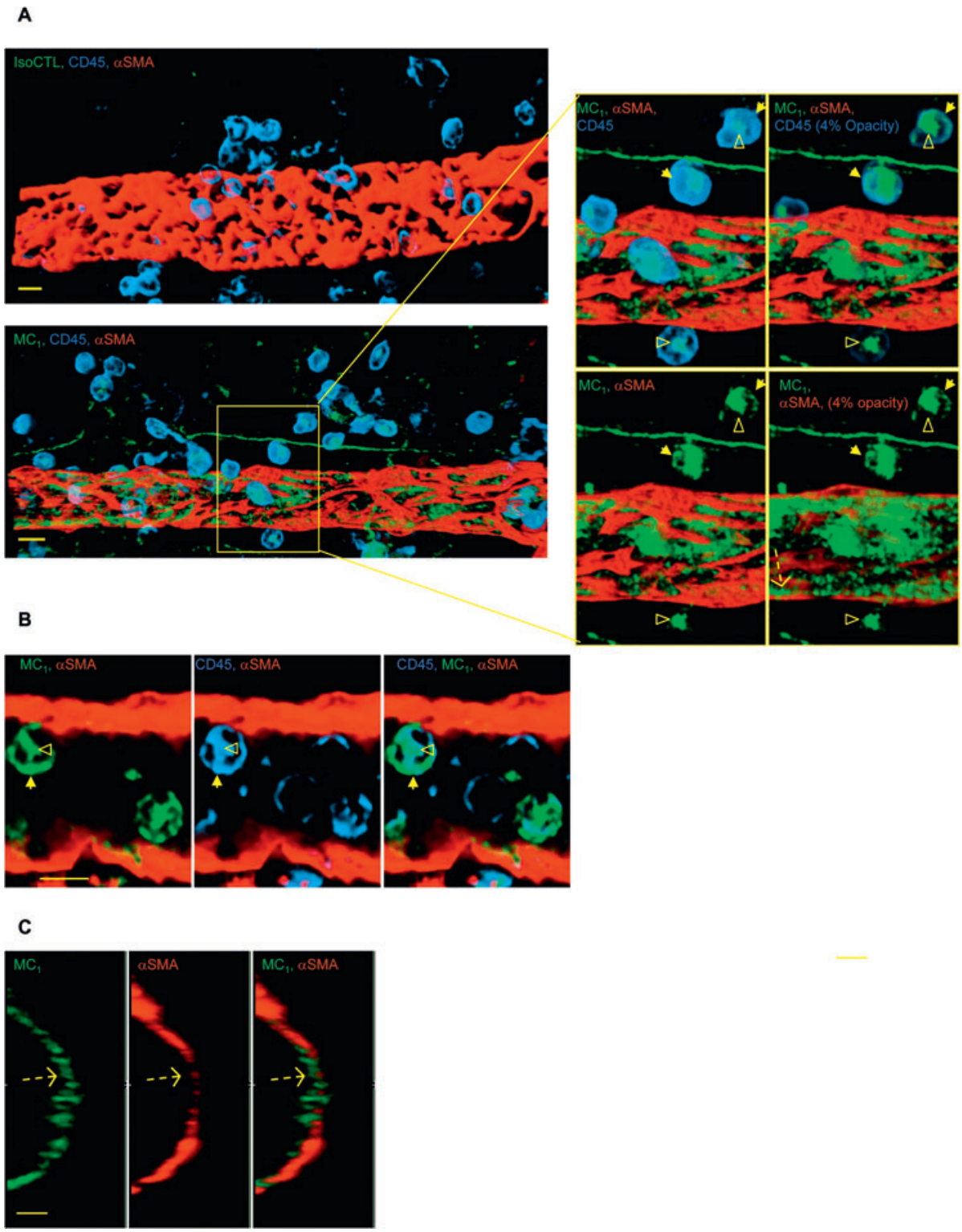

Figure 4 MC_1 receptor immunoreactivity in the inflamed mesentery. Confocal analyses of mesenteric tissue samples of WT mice. Tissues were obtained 90 min post-reperfusion, as detailed in Figure 1. There is an association between MC_1 receptor immunoreactivity and CD45-positive leucocytes. (A) Staining was performed with an irrelevant control Ab (top panel) or anti-MC_1 receptor Ab (bottom panel) in inflamed post-capillary venules. Images in the right panels are greater magnification of the region of interest showing expression of MC_1 receptors by leucocytes. (B). Longitudinal cross section (2 μm) of a vessel showing luminal leucocytes expressing MC_1 receptors. (C) Latitudinal cross section (1 μm) showing some degree of MC_1 receptor staining within the vessel wall (below the pericyte layer; dotted arrow). In some images, an opacity filter was used reducing the colour intensity in one channel (either for CD45 or α-SMA immunostaining) to highlight expression of MC_1 receptors within the leucocyte (open arrowhead), on the surface of the leucocyte (closed arrows) and on the luminal side of the vessel wall (dotted arrow) respectively. Data are representative of images obtained from tissues acquired from three mice. isoCTL, isotype control; αSMA, alpha-smooth muscle actin; MC_1, anti-MC_1 Ab; CD45, anti-CD45 Ab (se Methods for more details). Bar = 10 μm.

Table 1 Haemodynamic parameters in the mesenteric microcirculation of WT and inactive MC_1 e/e mice

Mouse genotype (procedure)	*Diameter (μm)*	*Cell flux (cells·min^{-1})*	*Wall shear rate (s^{-1})*
WT (sham)	25.3 ± 1.7	9.5 ± 1.5	400.5 ± 10.5
WT (35 + 90 IR)	27.2 ± 1.8	19.4 ± 0.9	280 ± 12.5
WT (35 + 90 IR) + BMS-470539	25.0 ± 1.6	20.4 ± 0.4	305 ± 10.5
Mutant MC_1 e/e (sham)	26.7 ± 1.2	8 ± 0.6	386.2 ± 32.0
Mutant MC_1 e/e (35 + 90 IR)	25.6 ± 3.8	20.5 ± 5.1	280.1 ± 18.2
Mutant MC_1 e/e (35 + 90 IR) + BMS-470539	28.6 ± 2.8	18.5 ± 3.1	300 ± 10.4

The diameter of the mesenteric vessels analysed in Figures 1 and 3 are summarized here, along with values for wall shear rate and cell flux. The mice were exposed to IR (35 min of ischaemia and 90 min of reperfusion). As indicated, BMS-470539 was given at a dose of 18.47 mg·kg^{-1} i.v. immediately before ischaemia. Data are mean ± SEM from six animals per group. For cell flux and wall shear rate, all IR values are significantly different from respective sham ($P < 0.01$).

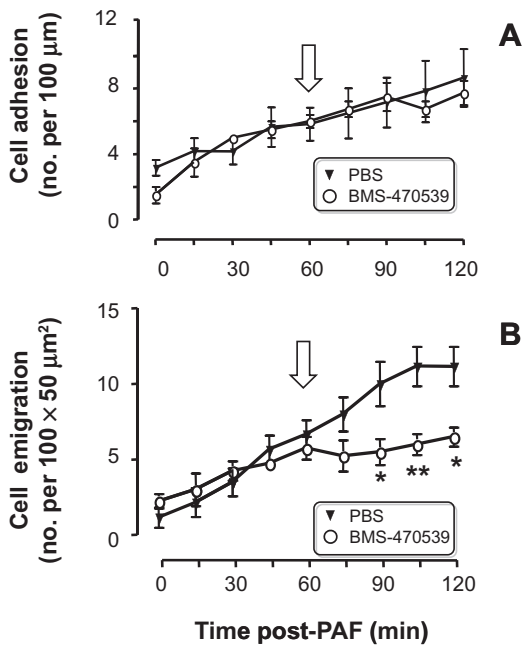

Figure 5 BMS-470539 inhibits cell emigration in the cremasteric microcirculation activated by PAF superfusion. WT mouse cremasteric microcirculation was superfused with buffer containing PAF (100 nM), and 1 min recordings were made every 15 min up to 120 min. After 60 min, buffer (100 μL) or BMS-470539 (18.57 mg·kg^{-1}) was given i.v. and cellular responses in the inflamed post-capillary venule determined after further 60 min. Cellular reactivity was monitored as cell adhesion (A) and emigration (B). Data are mean ± SEM of six mice per group. *$P < 0.05$, **$P < 0.01$ versus respective buffer value.

Table 2 Haemodynamic parameters in the mouse cremasteric microcirculation

Mouse (treatment)	*Cell flux (cells·min^{-1})*	*Wall shear rate (s^{-1})*
WT (PAF 30 min)	12.80 ± 2.9	426.66 ± 59.2
WT (PAF 60 min)	19.20 ± 2.4	446.66 ± 57.6
WT (PAF + buffer 90 min)	23.00 ± 5.0	465.55 ± 63.2
WT (PAF + buffer 120 min)	20.80 ± 4.5	553.33 ± 84.7
WT (PAF + BMS-470539 90 min)	17.60 ± 4.9	536.66 ± 84.1
WT (PAF + BMS-470539 120 min)	18.00 ± 4.8	549.99 ± 56.3

Values for wall shear rate and cell flux of experiments shown in Figure 5 are summarized here. Diameters of post-capillary venules (20–30 μm) are not shown, as they did not change over time. The mouse cremaster was superfused with 100 nM PAF (0–60 min), then either buffer (100 μL) or BMS-470539 (18.57 mg·kg^{-1}) was given i.v., and parameters monitored for another 60 min (120 min post-PAF superfusion). Data are mean ± SEM of six WT animals per group.

synthetic – peptides may become fruitful in the near future (Gonzalez-Rey *et al.*, 2007). A wealth of evidence indicates that α-MSH, a pan-agonist to all MC receptors, except MC_2, produces potent tissue-protective and anti-inflammatory effects in rodents, as well as in systems with human cells and samples (Catania *et al.*, 2004; Getting, 2006; Brzoska *et al.*, 2008). In line with the potential exploitation of other endogenous anti-inflammatory pathways and targets for the development of novel anti-inflammatory drugs (Gilroy *et al.*, 2004; Gonzalez-Rey *et al.*, 2007; Serhan *et al.*, 2007), it is possible that activation of MC receptors would have a lower burden of side effects as it would be mimicking the mechanisms employed endogenously to terminate inflammatory events.

In the area of MCs and MC receptors in inflammation, attention has been focused on two members of this subfamily of G-protein-coupled receptors, namely MC_1 and MC_3 receptors. Initial pharmacological studies have implied a strong involvement of MC_1 receptors in the anti-inflammatory properties of α-MSH, ACTH and other MCs (Lipton *et al.*, 1999; Catania *et al.*, 2000). Subsequently, MC_3 receptors were shown to be expressed on resident macrophages, and be activated to elicit inhibition in several settings of acute inflammation (Getting *et al.*, 1999; 2002). Therefore, from a drug development perspective, there is a dual opportunity of developing either MC_1 or MC_3 receptor-selective agonists. Very few studies have assessed the pathophysiological roles of either MC_1 or MC_3 receptors, for instance, studying the phenotype of transgenic mice.

The recessive yellow e/e mouse, which bears inactive MC_1 receptors (Robbins *et al.*, 1993), did not display differences in the acute inflammatory reaction to zymosan or other inflammogens, both for leucocyte recruitment and cytokine production (Getting *et al.*, 2003; 2006). On the other hand, using two distinct models of colitis, disease exacerbation was shown in this mouse colony, favouring the possibility that MC_1 receptors might be activated, or its endogenous agonists produced, in more chronic inflammatory conditions (Maaser *et al.*, 2006). This conclusion is supported by the data presented here, where the recessive yellow e/e mouse was able to mount a leucocytic response in the vasculature, very similar to that noted in WT controls. These findings obtained from studies in different microvascular beds and stimulated by two different stimuli, in which cell adhesion and emigration were

promoted by an IR procedure or PAF superfusion. Lack of involvement of MC_1 receptors in these events may be due to the lack of generation of its endogenous selective ligands. Of importance, the receptor was highly expressed in inflamed tissues most notably by adherent and emigrated (or resident) leucocytes.

The scenario, and ensuing conclusions, are quite different for MC_3 receptors. Vascular inflammation is exacerbated in MC_3 receptor null mice, as recently reported (Leoni *et al.*, 2008). Application of the IR procedure, identical to that used in the present study, leads to marked cell adhesion and emigration with values >50% augmented with respect to WT mice. This increased vascular reactivity in MC_3 receptor null mice is associated with, or is consequent to, an augmented tissue generation of pro-inflammatory chemokines, probably produced by tissue resident mast cells and macrophages (Ajuebor *et al.*, 1999; Tailor *et al.*, 1999). In line with the above hypothesis, it is possible that under these inflammatory conditions, endogenous selective MC_3 receptor ligands are produced and active. Peripheral generation of the pro-opiomelanocortin gene product is now an accepted finding (Gonzalez-Rey *et al.*, 2007; Brzoska *et al.*, 2008); however, the possibility that this polypeptide might be processed in a tissue-specific manner is yet to be tested and proven. Another theoretical explanation for the engagement of MC_3, and not MC_1 receptors, in the early (from 0 to 4 h) tissue inflammatory response could lie in the fact that post-translational modifications may occur, changing the affinity or the susceptibility to activation of one or the other MC receptor. Future studies will address these possibilities, which clearly are not mutually exclusive.

Our observation that MC_1 receptor expression is detectable on inflammatory cells is of importance, as such a phenomenon has often been indicated using molecular approaches, but rarely in whole tissues, and in such a clear-cut manner. Our confocal analyses allowed prompt detection of MC_1 receptor on CD45+ cells, likely to be associated with cells adherent to the vascular wall, as well as with cells that had migrated into the tissue. In our previous study, we employed immunohistochemistry to detect MC_1 and MC_3 receptor immunoreactivity in extravasated neutrophils and macrophages within the post-IR mesenteric tissue (Leoni *et al.*, 2008). Here, we demonstrated that adherent leucocytes are MC_1 receptor positive. In line with previous reports (Scholzen *et al.*, 2003), a weak expression of MC_1 receptor on vascular endothelium could be observed; this is congruent with the ability of MC peptides to down-regulate endothelial cell expression of cell adhesion molecules (Scholzen *et al.*, 2003). Here, we have not evaluated the effects of BMS-470539 on endothelial cells, and therefore cannot exclude a contributing role on modulation of endothelial cell adhesion molecules. It is possible that in addition to the effects observed on circulating leucocytes, an effect on the endothelium might occur in our experimental settings, which would aid in eliciting a reduction in the extent of leucocyte emigration.

Use of the recessive yellow e/e mouse indicated that activation of MC_1 receptors mediates, on its own, the anti-inflammatory effects of BMS-470539. In the mesenteric post-IR tissue, this selective MC_1 receptor agonist prevented not only cell emigration, but also tissue generation of CCL2 and CXL1. These two chemokines are major effectors in recruiting neutrophils and monocytes in the early phases of the inflammatory response, being promptly produced by tissue mast cells and macrophages (Ajuebor *et al.*, 1999). It is likely that their role is to promote adhesion of rolling leucocytes, a prerequisite to subsequent emigration. With the IR protocol, we could not discriminate between a direct effect of BMS-470539 on cell adhesion and emigration, or whether the latter was secondary to a lowered tissue generation of pro-inflammatory chemokines. To address this further, we had to apply a protocol that could allow monitoring of the development of leucocyte reactivity in the vasculature. The data generated here highlight that inhibition of chemokine expression leads to the reduction in leucocyte recruitment observed within these models. Previous studies have shown that MC peptides can down-regulate the expression of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and TNF-α, as well as chemokines including IL-8 (Luger *et al.*, 1997; Brzoska *et al.*, 2008). Moreover, it is also established that following this initial inhibition of pro-inflammatory chemokine and cytokine release, MC receptor agonists activate a delayed induction of anti-inflammatory pathways provoking an increase in IL-10 and haem oxygenase 1 expression (Lam *et al.*, 2005; 2006).

Superfusion of PAF onto the microvasculature is an established protocol (Kubes *et al.*, 1990; Zimmerman *et al.*, 1994; Chatterjee *et al.*, 2005) suitable to study alterations in the microcirculation of stable preparations of intravital microscopy. The cremaster preparation satisfies this requisite (Gavins and Chatterjee, 2004). PAF application is known to produce direct activation of the intravascular leucocytes and the endothelium, provoking selectin-mediated rolling, followed by cell adhesion and emigration, along the established model for leucocyte recruitment (Ley *et al.*, 2007). Besides testing the effects of BMS-470539 against a different stimulus and in another vasculature, we chose to apply the compound with a therapeutic protocol. Given at the anti-inflammatory dose of 18.47 $mg \cdot kg^{-1}$ i.v., BMS-470539 provoked rapid inhibition of the process of cell emigration, with no particular efficacy on the mounting of the cell adhesion response. This effect was remarkable because no further increase of the number of emigrated leucocytes was evident as early as 10 min after compound administration.

Altogether, these data indicate that MC_1 receptor activation in an inflamed microvasculature, as specifically achieved here with BMS-470539, can produce at least two distinct outcomes: (i) inhibition of cell adhesion, possibly as a consequence of a marked attenuation of the generation of pro-inflammatory chemotactic factors; and (ii) inhibition of cell emigration along a mechanism that can be distinct from the actions on cell adhesion, most likely a consequence of MC_1 receptor activation on the adherent leucocytes (as we have shown for the first time). In both models used, and in contrast to the above, activation of MC_1 receptors did not influence the process of leucocyte rolling.

In conclusion, this study corroborates the validity of developing selective MC_1 receptor ligands, peptides or new chemical entities like BMS-470539, to counteract aberrant inflammatory responses, including those characteristic of an IR pathological scenario, such as those observed after thrombus-provoked stroke or organ transplantation.

Acknowledgements

This study was funded by the British Heart Foundation (studentship FS/05/078/19406). Research on MC receptor biology (M.P.) is funded by the Arthritis Research Campaign UK. This work forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, which is supported and funded by the National Institute of Health Research. Other research (S.N.) is funded by the Wellcome Trust (081172/Z/06/Z).

Conflict of interest

M.P. is involved in a collaborative project on novel MCs with Action Pharma A/S (Holte, Denmark).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 MC1 receptor immunoreactivity in the inflamed cremaster. Confocal analyses of cremasteric tissue samples of WT mice. Tissues were obtained 120 min post-PAF superfusion, as detailed in Figure 5. MC_1 receptor immunoreactivity was associated with CD45-positive leucocytes. Staining with an irrelevant control Ab (A) or anti-MC_1 Ab (B) in inflamed post-capillary venules. CD45 staining is demonstrated in (C) and (D). Data are representative of images produced acquired from tissues from three mice. isoCTL, isotype control; MC_1, anti-MC_1 Ab; CD45, anti-CD45 Ab (see Methods for more details).

Figure S2 No differences in the reactivity of the cremasteric microcirculation of MC_1 e/e recessive yellow mice during PAF superfusion. WT and MC_1 recessive yellow e/e mouse cremasteric microcirculation was superfused with buffer containing PAF (100 nM), and 1 min recordings were made every 15 min up to 120 min. Cellular reactivity was monitored as cell adhesion (A) and emigration (B). Data are mean ± SEM of six mice per group.

Figure S3 BMS-470539 does not inhibit cell emigration in the recessive yellow e/e cremasteric microcirculation activated by PAF superfusion. Recessive yellow e/e mouse cremasteric microcirculation was superfused with buffer containing PAF (100 nM), and 1 min recordings were made every 15 min up to 120 min. After 60 min, buffer (100 μL) or BMS-470539 (18.57 $mg \cdot kg^{-1}$) was given i.v., and cellular responses in the inflamed post-capillary venule determined after further 60 min. Cellular reactivity was monitored as cell adhesion (A) and emigration (B). Data are mean ± SEM of six mice per group.

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