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Role of neutrophils in exacerbation of brain injury after focal cerebral ischemia in hyperlipidemic mice

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Abstract

Background and Purpose—Inflammation-related co-morbidities contribute to stroke-induced immune responses and brain damage. We previously showed that hyperlipidemia exacerbates ischemic brain injury, which is associated with elevated peripheral and cerebral granulocyte numbers. Herein, we evaluate the contribution of neutrophils to the exacerbation of ischemic brain injury.

Methods—Wildtype mice fed with a normal chow and ApoE knockout mice fed with a high cholesterol diet were exposed to middle cerebral artery occlusion (MCAO). CXCR2 was blocked using the selective antagonist SB225002 (2 mg/kg) or neutralizing CXCR2 antiserum. Neutrophils were depleted using an anti-Ly6G antibody. At 72 hours post-ischemia immunohistochemistry, flow cytometry and real-time PCR was performed to determine cerebral tissue injury and immunological changes in the blood, bone marrow and brain. Functional outcome was assessed by accelerated rota rod and tight rope tests at 4, 7 and 14 days post-ischemia.

Results—CXCR2 antagonization reduced neurological deficits and infarct volumes that were exacerbated in hyperlipidemic ApoE$^{-/-}$ mice. This effect was mimicked by neutrophil depletion. Cerebral neutrophil infiltration and peripheral neutrophilia, which were increased upon ischemia in hyperlipidemia, were attenuated by CXCR2 antagonization. This downscaling of neutrophil responses was associated with increased neutrophil apoptosis and reduced levels of CXCR2, iNOS and NOX2 expression on bone marrow neutrophils.

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Disclosures

None.

Conclusion—Our data demonstrate a role of neutrophils in the exacerbation of ischemic brain injury induced by hyperlipidemia. Accordingly, CXCR2 blockade, which prevents neutrophil recruitment into the brain, might be an effective option for stroke treatment in patients suffering from hyperlipidemia.

Keywords

CXCR2; neutrophil; stroke; hyperlipidemia

Introduction

Inflammation is involved in stroke-induced brain damage[1]. Besides, local inflammatory processes in the ischemic brain, stroke also provokes peripheral immune responses, which influence secondary lesion growth and thus modulate long-term outcome. However, difficulties remain with regard to the translation of inflammation-targeting therapeutic approaches from pre-clinical to clinical studies[2], which might be due to a neglect of co-morbidities such as hyperlipidemia that is frequently shown in stroke patients. In fact, using dietary and genetically-induced models of hyperlipidemia previous studies demonstrated increased ischemic brain injury in hyperlipidemic mice[3–5]. Underlying mechanisms involve alterations of the blood brain barrier[3]. However, in addition to vascular pathology hyperlipidemia triggers granulocytosis[4, 6] and therefore adds another complexity to stroke-induced pathophysiology in hyperlipidemic mice hampering translation of experimental findings into clinical practice. Emerging experimental and clinical evidences suggest that inflammatory factors outside the brain markedly influence stroke susceptibility and outcome[7]. Thus, we have previously shown that the combination of genetically and dietary-induced hyperlipidemia leads to increased cerebral ischemic tissue injury which is accompanied by elevated levels of cerebral and circulating granulocytes, albeit the causal link and the functional significance of these observations still remained elusive[4].

Early infiltration of polymorphonuclear neutrophils that is preceded by activation of endothelial cells and expression of chemokines and adhesion molecules is a major hallmark of post-ischemic inflammation[8]. Several reports including our own suggest a detrimental role of brain infiltrating neutrophils in ischemic tissue damage, e.g. by releasing oxygen radicals and inflammatory mediators[9–12]. Besides adhesion molecules, brain-derived chemokines facilitate immune cell transmigration into the inflamed brain tissue. Neutrophils are recruited through the specific interaction of CXCL1 and CXCL2/3, which are strongly upregulated in ischemic brains, with the neutrophil-specific receptor CXCR2[13]. In addition to enrollment of neutrophils, CXCR2 is supposed to mediate neutrophil release from the bone marrow into the blood in response to inflammatory challenges[14].

In view of our earlier findings[4], we were interested whether neutrophils contribute to the exacerbation of ischemic brain injury induced by hyperlipidemia. Therefore, we investigated how the antagonization of the neutrophil-specific chemokine receptor CXCR2 by means of a selective pharmacological inhibitor or a neutralizing CXCR2 antiserum influenced ischemic brain injury and functional recovery in hyperlipidemic ApoE$^{-/-}$ mice, furthermore

investigating consequences of CXCR2 antagonization on peripheral neutrophil homeostasis and phenotype as well as on cerebral neutrophil infiltration.

Materials and Methods

Animals and group allocation

Experiments were performed in accordance to the ARRIVE guidelines and the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals with local government approval. Male 7–8 weeks old wildtype (C57BL/6J, Harlan) and ApoE$^{-/-}$ mice, which were generated on the same C57BL/6 background, were either fed with a normal chow or a cholesterol rich chow (Western Diet; TD.88137 Adjusted Calories Diet, Harlan Laboratories) for 6 weeks and submitted to 20 min of left-sided middle cerebral artery occlusion (MCAO) or sham surgery. Animals were randomly attributed to treatment paradigms, and experimenters were blinded at all stages of interventions and data analysis. The selective CXCR2 antagonist SB225002 (2 mg/kg, Merck, Darmstadt) or vehicle (1% DMSO in phosphate buffered saline (PBS)) was injected intraperitoneally (i.p.) at 0, 24 and 48 hours post-ischemia. In other experiments, CXCR2 was specifically blocked by i.p. injection of a neutralizing rabbit anti-CXCR2 serum (300 µl) at 0 hours, 24 hours and 48 hours post-ischemia[15]. In the latter studies, normal rabbit serum (NRS) served as control. In some experiments, neutrophils were depleted by i.p. injection of 200 µg anti-mouse Ly6G (Clone 1A8, BioXcell) 24 hours before and 24 hours after ischemia. In these experiments, 200 µg of an isotype control antibody (Clone 2A3, BioXcell) was delivered as control[11]. A total of 352 male mice (184 C57Bl/6J, 168 ApoE$^{-/-}$) were examined. These mice were randomly assigned to the following groups: wildtype normolipidemic / sham / vehicle (n=18), wildtype normolipidemic / MCAO / vehicle (n=47), wildtype normolipidemic / sham / CXCR2 antagonist (n=18), wildtype normolipidemic / MCAO / CXCR2 antagonist (n=47), ApoE$^{-/-}$ hyperlipidemic / sham / vehicle (n=18), ApoE$^{-/-}$ hyperlipidemic / MCAO / vehicle (n=39), ApoE$^{-/-}$ hyperlipidemic / sham / CXCR2 antagonist (n=18), ApoE$^{-/-}$ hyperlipidemic / MCAO / CXCR2 antagonist (n=39), wildtype normolipidemic / MCAO / NRS (n=9), wildtype normolipidemic / MCAO / anti-CXCR2 (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / NRS (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / anti-CXCR2 (n=9), wildtype normolipidemic / MCAO / isotype / vehicle (n=9), wildtype normolipidemic / MCAO / isotype / CXCR2 antagonist (n=9), wildtype normolipidemic / MCAO / anti-Ly6G / vehicle (n=9), wildtype normolipidemic / MCAO / anti-Ly6G / CXCR2 antagonist (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / isotype / vehicle (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / isotype / CXCR2 antagonist (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / anti-Ly6G / vehicle (n=9), ApoE$^{-/-}$ hyperlipidemic / MCAO / anti-Ly6G / CXCR2 antagonist (n=9). The groups were further divided into four subgroups: a) to assess functional deficits at 4, 7 and 14 days post-ischemia (dpi), b) to determine infarct volumes and for immunohistochemistry, c) to quantify leukocyte subsets in blood, bone marrow and brain at 3 dpi and d) to analyze mRNA expression via real-time PCR in bone marrow neutrophils at 3 dpi. A detailed description on subgroup allocation and exclusion criteria is given in the Data Supplement.

Induction of focal cerebral ischemia

Induction of stroke was performed using the intraluminal monofilament occlusion model as described previously[4]. A detailed description is given in the Data Supplement.

Analysis of post-stroke motor coordination deficits

Assessment of motor coordination deficits was performed on days 4, 7 and 14 using the rota rod and the tight rope test as previously described[16]. Details are given in the Data Supplement.

Analysis of post-ischemic tissue injury and immunohistochemistry

For infarct volume measurement and immunohistochemical analysis, mice were transcardially perfused with ice-cold PBS at 72 hours post-ischemia. Brains were removed and fresh frozen on dry ice. To determine infarct volumes 20 μm cryostat sections (every 400 μm between +2 mm up to −4.4 mm from bregma) were stained with cresyl violet. For assessment of cell death, endothelial activation, neutrophil infiltration and oxidative DNA damage, cryostat sections taken at the level of bregma were used for immunohistochemistry according to published protocols[4, 17]. A detailed description of stainings and quantifications is given in the Data Supplement.

Processing of peripheral blood, bone marrow and brain tissues for flow cytometry analysis

Isolation of single cell suspension for flow cytometry analysis was performed as previously described[4]. Briefly, animals were euthanized by i.p. injections of chloralhydrate (200 mg/kg body weight). Blood specimens were collected into ethylenediaminetetraacetate (EDTA) coated collection tubes by puncture of the inferior *vena cava* followed by transcardial perfusion with ice-cold PBS. Brains were dissected and hemispheres divided into ipsi- and contralesional parts. Bone marrow from femurs and tibiae was flushed with PBS. A detailed description of further single cell isolation, staining procedures, antibody cocktails and gating strategies is given in the Data Supplement (Supplemental Methods, Supplemental Table I).

Gene expression analysis of sorted neutrophils by real time PCR

For gene expression studies, *ex vivo*-isolated bone marrow cells were sorted for the neutrophil specific antigen Ly6G using magnetic activated cell sorting (MACS®). Total RNA was extracted from cell lysates (Promega, Madison) transcribed to cDNA followed by real time PCR. Details on the experimental conditions and measurements as well as primer sequences are given in the Data Supplement (Supplemental Methods, Supplemental Table II).

Statistics

Numbers of animals to detect infarct size (n=9) and functional deficits (n=12) were determined via a priori sample size calculations (effect size *f* [by ANOVA] of 0.6 for infarct size, and 0.5 for functional deficits with α=0.05, β=0.2). Results are presented as means ± SD. Differences between 2 groups were assessed by the 2-tailed Student *t* test. Differences across multiple groups were analyzed using 2- or 3-way ANOVAs with phenotype (wildtype

normolipidemic vs. ApoE$^{-/-}$ hyperlipidemic), experimental intervention (sham vs. MCAO) or treatment (vehicle vs. CXCR2 antagonist) as independent factors followed by post hoc Bonferroni tests for pairwise comparisons. In all analyses, $p<0.05$ was considered statistically significant.

Results

CXCR2 inhibition promotes functional recovery and reduces ischemia-induced cerebral tissue injury in hyperlipidemic ApoE$^{-/-}$ mice

We and others have recently shown that induction of hyperlipidemia by means of a cholesterol-rich chow is associated with exacerbation of ischemic injury in Apo$^{-/-}$ mice[4, 5]. Motor-coordination deficits assessed in the rota rod and the tight rope test, which were aggravated by hyperlipidemia in ApoE$^{-/-}$ mice, were markedly improved by CXCR2 antagonization up to 14 days post-ischemia (Fig. 1A, B). Administration of the selective CXCR2 inhibitor SB225002 did not affect ischemic brain injury in normolipidemic wildtype mice, but reversed the increased brain injury in hyperlipidemic ApoE$^{-/-}$ mice (Fig. 1C, D). This effect was mimicked by a neutralizing CXCR2 anti-serum[15] (Fig. 2A). To exclude the possibility that 72 hours was too late to detect differences in brain injury of normolipidemic mice, we also analyzed infarct volume at 24 hours post-ischemia. Again, infarct volume was not altered by CXCR2 deactivation (Supplemental Fig. I).

The neuroprotective effect of CXCR2 antagonization is abrogated in neutrophil-depleted hyperlipidemic ApoE$^{-/-}$ mice

To test whether neuroprotective effects in hyperlipidemic mice were attributed to specific effects on neutrophils, we assessed infarct volume in ischemic mice, which had been neutrophil-depleted using a specific anti-Ly6G (1A8) antibody[11] and in which CXCR2 was inhibited with SB225002 (Fig. 2B). Neutrophil depletion led to a significant reduction of infarct volume in hyperlipidemic ApoE$^{-/-}$ mice but in normolipidemic wildtype mice (Fig. 2C). Of note, administration of the CXCR2 antagonist did not further reduce infarct volume in neutrophil-depleted mice (Fig. 2C), indicating that neuroprotection by SB225002 can be assigned to a direct effect on CXCR2-expressing neutrophils.

Inhibition of CXCR2 reduces neutrophil infiltration without affecting endothelial activation

To analyze whether brain neutrophil invasion was blocked by pharmacological CXCR2 antagonization, we quantified cerebral neutrophils infiltrated in ipsi- and contralateral hemispheres by flow cytometry. The CXCR2 antagonist SB225002 decreased neutrophil counts in ischemic hemispheres of ApoE$^{-/-}$ mice on Western diet and wildtype mice on normal diet (Fig. 3A). Recruitment of other immune cell subsets was not significantly modulated by CXCR2 inhibition (Supplemental Fig. II), suggesting a selective interaction with neutrophils. In order to exclude potential confounders related to differences in lesion size we quantified the regional densities of neutrophils within the lesion rim by Ly6G immunohistochemistry and furthermore evaluated their localization with respect to brain capillaries in co-stainings with the pan-endothelial marker CD31. Although previous studies had shown a predominant association of neutrophils with ischemic brain vessels[18], we observed a significant proportion of neutrophils in the brain parenchyma for all investigated

groups (Fig. 3B), closely in line with recent reports of others and us[11, 19]. The local distribution of neutrophils was not significantly modulated by hyperlipidemia or by administration of the CXCR2 antagonist SB225002 (Fig. 3B top middle). Distances of intra-parenchymal neutrophils to the most adjacent vessel were also not changed (Fig. 3B, top right). Therefore, we quantified the total number of neutrophils (vessel-associated and intra-parenchymal) revealing significantly reduced numbers of neutrophils in the injured brain of ischemic hyperlipidemic ApoE$^{-/-}$ mice treated with SB225002 compared to vehicle-treated mice and ischemic normolipidemic wildtype mice (Fig. 3B, bottom). To avoid false interpretation due to unspecific effects of SB225002 on BBB characteristics, which might have contributed to inhibition of neutrophil infiltration and/or tissue injury, we also performed a detailed analysis of blood brain barrier (BBB) integrity and endothelial activation. Except of an increased BBB permeability (Supplemental Fig. IIIA) and increased ICAM-1 and VCAM-1 expression (Fig. 3C, D) in ischemic hyperlipidemic ApoE$^{-/-}$ mice compared to normolipidemic wildtype mice, no significant changes were induced by systemic SB225002 administration. The overall number of vessels was affected neither by hyperlipidemia nor by CXCR2 antagonization (Supplemental Fig. IIIB). Therefore, reduced neutrophil infiltration might be directly attributed to the CXCR2-inhibitory effect of SB225002.

Peripheral neutrophilia in ischemic hyperlipidemic ApoE$^{-/-}$ mice is reduced by CXCR2 antagonization

In addition to chemotactic activity, we examined whether modulation of CXCR2 changes the number of peripheral neutrophils. Analysis of the absolute viable neutrophil counts demonstrated that focal cerebral ischemia induced an increase in circulating and bone marrow neutrophils in hyperlipidemic ApoE$^{-/-}$ mice, which was inhibited by SB225002 (Fig. 4). These data suggest that neutrophil homeostasis is altered in the bone marrow, in addition to peripheral blood. Of note, neither focal cerebral ischemia nor CXCR2 antagonization by SB225002 influenced the number of blood and bone marrow neutrophils in normolipidemic wildtype mice (Fig. 4). In addition to an ischemia-independent overall increase in myeloid cells in the blood and reduced lymphocyte numbers in the bone marrow of hyperlipidemic ApoE$^{-/-}$ mice, we observed an ischemia-induced reduction in lymphocyte subsets in all experimental groups (Supplemental Fig. IV). However, none of these immune cell subsets were regulated by the CXCR2 inhibitor, suggesting a selective interaction with neutrophils (Supplemental Fig. IV).

Stroke-induced reduction of neutrophil apoptosis and increase in CXCR2 expression on bone marrow neutrophils of hyperlipidemic ApoE$^{-/-}$ mice is reversed by antagonization of CXCR2

In order to explain the SB225002-mediated reduction in viable neutrophil counts in the periphery we next evaluated neutrophil survival by quantifying the proportion of dead neutrophils by flow cytometry using propidium iodide (PI). Upon CXCR2 antagonization, ischemic hyperlipidemic ApoE$^{-/-}$ mice revealed an increased proportion of dead neutrophils in the bone marrow (Fig. 5A), suggesting that SB225002 counteracts the ischemia-induced increase of neutrophil survival in hyperlipidemic ApoE$^{-/-}$ mice. In normolipidemic wildtype mice, on the other hand, neither ischemia nor CXCR2 deactivation modulated the proportion

of dead neutrophils (Fig. 5A). To exclude any confounding effects by false positive detection rarely associated with PI staining we quantified the proportion of AnnexinV positive bone marrow neutrophils in ischemic hyperlipidemic ApoE$^{-/-}$ mice either treated with vehicle or with the CXCR2 antagonist SB225002. Confirming PI measurements, increased numbers of apoptotic cells were noted in SB225002 treated mice (Fig. 5B).

Next, we wondered whether CXCR2 expression on neutrophils was affected by hyperlipidemia or ischemia. Flow cytometry and real time PCR revealed that CXCR2 protein and mRNA expression on bone marrow neutrophils were increased upon ischemia in hyperlipidemic ApoE$^{-/-}$ mice, which were reversed through CXCR2 deactivation by SB225002 (Fig. 5C, D). In normolipidemic wildtype mice, neither ischemia nor CXCR2 antagonization affected CXCR2 expression on bone marrow neutrophils (Fig. 5C, D).

The CXCR2 antagonist SB225002 modulates oxidative stress-related enzyme expression in bone marrow neutrophils and reduces oxidative damage in ischemic brains of hyperlipidemic ApoE$^{-/-}$ mice

To further eludicate mechanisms for the differential effects of CXCR2 inhibition in normolipidemic and hyperlipidemic mice we finally investigated whether oxidative stress-related enzyme expression was modulated by hyperlipidemia, ischemia and CXCR2 antagonization. mRNA expression analysis of the inflammation-associated inducible nitric oxide synthase (iNOS) in sorted bone marrow neutrophils demonstrated that focal cerebral ischemia induces a strong increase in hyperlipidemic ApoE$^{-/-}$ but not in normolipidemic wildtype mice, which was reversed by CXCR2 blockade (Fig. 6A). Moreover, a significant ischemia-induced decrease of NADPH oxidase 2 (NOX2) expression detected in normolipidemic wildtype mice was absent in hyperlipidemic ApoE$^{-/-}$ mice. However, CXCR2 deactivation by SB225002 reduced NOX2 expression in ischemic hyperlipidemic ApoE$^{-/-}$ mice to levels of ischemic normolipidemic wildtype mice (Fig. 6B). We further observed an overall reduced expression of the anti-oxidative enzymes catalase (CAT) and superoxide dismutase 2 (SOD2) in ischemic and non-ischemic hyperlipidemic ApoE$^{-/-}$ mice that was not influenced by CXCR2 inhibition (Supplemental Fig. V). Taken together, these data suggest that hyperlipidemia combined with focal cerebral ischemia promote an inflammatory and reactive oxygen species (ROS) producing phenotype in ischemic hyperlipidemic ApoE$^{-/-}$ mice which is partially counterbalanced by CXCR2 deactivation. In light of the presented results we further analyzed oxidative DNA damage through immunohistochemical detection of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) as an indicator of oxidative stress. Quantification of 8-oxo-dG positive cells in ischemic brain tissue revealed that the CXCR2 antagonist SB225002 reduced increased levels of oxidative DNA damage in hyperlipidemic ApoE$^{-/-}$ mice, while normolipidemic wildtype mice exhibited an overall low density of 8-oxo-dG positive cells which was not modulated by the CXCR2 antagonist (Fig. 6C).

Discussion

Systemic inflammation is linked to stroke occurrence and severity. However, translation from bench to bedside targeting the immune system to prevent stroke or diminish damage

has failed so far. One critical issue regarding the lack of successful translation is that underlying inflammation associated with co-morbidity factors such as hyperlipidemia have been broadly neglected in most pre-clinical trials. We have previously shown that increased ischemic brain damage in hyperlipidemic mice coincides with increased cerebral and peripheral granulocyte numbers[4]. In the present study we provide the causal link between these observations by showing that pharmacological or antiserum-mediated antagonization of the neutrophil-specific chemokine receptor CXCR2 or anti-Ly6G antibody-induced neutrophil depletion restores functional outcome and reverses brain injury induced by hyperlipidemia, thereby unraveling the functional significance of neutrophils in the pathogenesis of ischemic brain injury. Interestingly, only hyperlipidemic mice were responsive to CXCR2 antagonization, cerebral neutrophil infiltration being strongly attenuated by CXCR2 inhibition in hyperlipidemic mice. This increased chemotactic activity of neutrophils in ischemic hyperlipidemic mice was most likely evoked by the increased CXCR2 expression on peripheral neutrophils, perhaps combined with the previously reported increase of its ligands CXCL1 and CXCL2 in ischemic brains of hyperlipidemic mice[4]. Chemotactic receptors, such as CXCR2 are involved in interactions with endothelial cells and platelets, which play an important role in atherosclerosis development[20] and which regulate neutrophil crawling in inflamed vessels[21]. Thus brain platelet-neutrophil interactions might be associated with the initiation of neutrophil infiltration into the ischemic brain in hyperlipidemia.

In addition to enhanced chemoattraction to the brain, elevated cerebral neutrophil counts in hyperlipidemic stroke mice might partially result from the overall increased number of circulating neutrophils induced by ischemia. Importantly, CXCR2 antagonization by SB225002 reversed this peripheral neutrophilia. Since increased circulating neutrophils were paralleled by increased bone marrow neutrophils, elevation of blood neutrophils caused by a CXCR2-dependent release from the bone marrow to the circulation[14] seems unlikely. Instead, stroke-induced survival of bone marrow neutrophils in hyperlipidemia was blocked by CXCR2 antagonization. Indeed, neutrophil viability has been shown to be regulated by chemokines in interaction with CXCR2[22]. Furthermore, CXCR2 antagonization increases neutrophil apoptosis in a concentration-dependent manner[23], supporting our concept of induction of neutrophil apoptosis by the CXCR2 antagonist SB225002. The lack of apoptosis induction in sham-operated and ischemic normolipidemic mice might be explained by the lower CXCR2 expression. Thus, a certain chemokine receptor expression and activation level appears to be required for neutrophil survival.

Although CXCR2 blockade resulted in reduced cerebral neutrophil entry both in normolipidemic and hyperlipidemic mice, only hyperlipidemic mice benefited from CXCR2 inhibition, suggesting that in addition to the total number, the phenotype of neutrophils determines the impact on stroke outcome. We show that focal cerebral ischemia induces a strong increase of iNOS and NOX2 in neutrophils of hyperlipidemic mice that is downregulated by CXCR2 inhibition. Both enzymes are involved in the generation of reactive oxygen/nitrogen species, one of the main effector functions of neutrophils in stroke pathology[24, 25]. Alterations of the neutrophil phenotype in ischemic hyperlipidemic mice were associated with an increased level of cerebral oxidative DNA damage, which was similarly reversed by CXCR2 deactivation as iNOS. These associated findings may indicate

that decreased neutrophil activation and ROS production through CXCR2 antagonization might present a potential mechanism underlying the observed neuroprotective effects in hyperlipidemic ischemic mice. This is further supported by a previous study reporting reduced myeloperoxidase activity of neutrophils after CXCR2 antagonization[23]. In light of previous studies suggesting a certain neutrophil plasticity similarly to macrophage plasticity distinguishing pro-inflammatory M1 and anti-inflammatory M2 macrophages[19, 26], increased levels of iNOS, a classical M1 marker, imply that the combination of brain ischemia and hyperlipidemia might induce a general switch of neutrophils to a pro-inflammatory N1 phenotype which is reversed by CXCR2 antagonization. Additional studies will be required to characterize the whole repertoire of classical M1/N1 and M2/N2 markers in this specific experimental setting.

Despite reduced cerebral neutrophil infiltration, normolipidemic mice were not protected by CXCR2 inhibition. This observation appears to differ from recent studies including our own demonstrating neuroprotection by neutrophil depletion and/or blocking neutrophil invasion[9–12]. The most likely explanation is the different severity of brain injury. While the latter studies induced 45 and 60 min MCAO we used a mild ischemia model leading to much smaller infarcts and a lesser degree of neutrophil accumulation, suggesting that a certain threshold of cerebral neutrophil counts needs to be exceeded to uncover the cells' pathogenic role. However, even if more severe injury models are used, several studies interfering with CXCR2 signaling and thereby reducing neutrophil infiltration failed to influence stroke outcome in normolipidemic wildtype mice[27–29]. But, most of these studies assessed stroke outcome at 24 hours post injury which might be too early to detect secondary neurodegeneration after the complex inflammatory cascade has fully established. Although neutrophils are considered to be the first invaders of the ischemic brain, secondary recruitment of neutrophils through IL-17 producing γδ T cells peaking at 3 days post injury has been suggested [30]. Therefore, it cannot be excluded to detect protection in a more severe stroke model at 3 to 7 days post-ischemia. In fact, administration of neutralizing CXCR2 serum in a severe transient MCAO model in normolipidemic wildtype mice results in significantly smaller infarct sizes 3 days after stroke[30]. On the other hand, Cuartero et al. demonstrated that neutrophil infiltration is maximal after 24 hours and that modification of neutrophil phenotype by rosiglitazone is associated with significantly reduced infarct volumes in normolipidemic mice at 24 hours post-ischemia[19] indicating that 72 hours might have been too late to detect the impact of CXCR2 inhibition in normolipidemia. In our study, infarct volume in normolipidemic mice was unaffected by CXCR2 antagonization at 24 hours and 72 hours post stroke, demonstrating that the observations made did not depend on the selection of the time window. Nevertheless, specific characteristics of ischemia models (e.g. distal vs. intraluminal) should carefully be considered, when evaluating observations in earlier studies[31].

In conclusion, our study suggests that the contribution of neutrophils to the development of ischemic brain injury depends on the pathophysiological state. Thus, neutrophils have a unique role in stroke pathogenesis in hyperlipidemia. Similarly, differential roles of immune cell subsets have previously been shown for $CCR2^+$ monocytes which are important for vascular stabilization in normolipidemic ischemic mice [32] but harmful in acute hyperlipidemia[5]. By showing that hyperlipidemic mice –unlike normolipidemic mice-

respond to the inhibition of the neutrophil-specific chemokine receptor CXCR2, we provide a potential therapeutic strategy for attenuating the exacerbation of ischemic injury induced by hyperlipidemia. In the evaluation of new therapies, pre-clinical and clinical studies should carefully consider inflammation-associated co-morbidities, namely hyperlipidemia, which, as we show, may alter the responsiveness of the ischemic brain tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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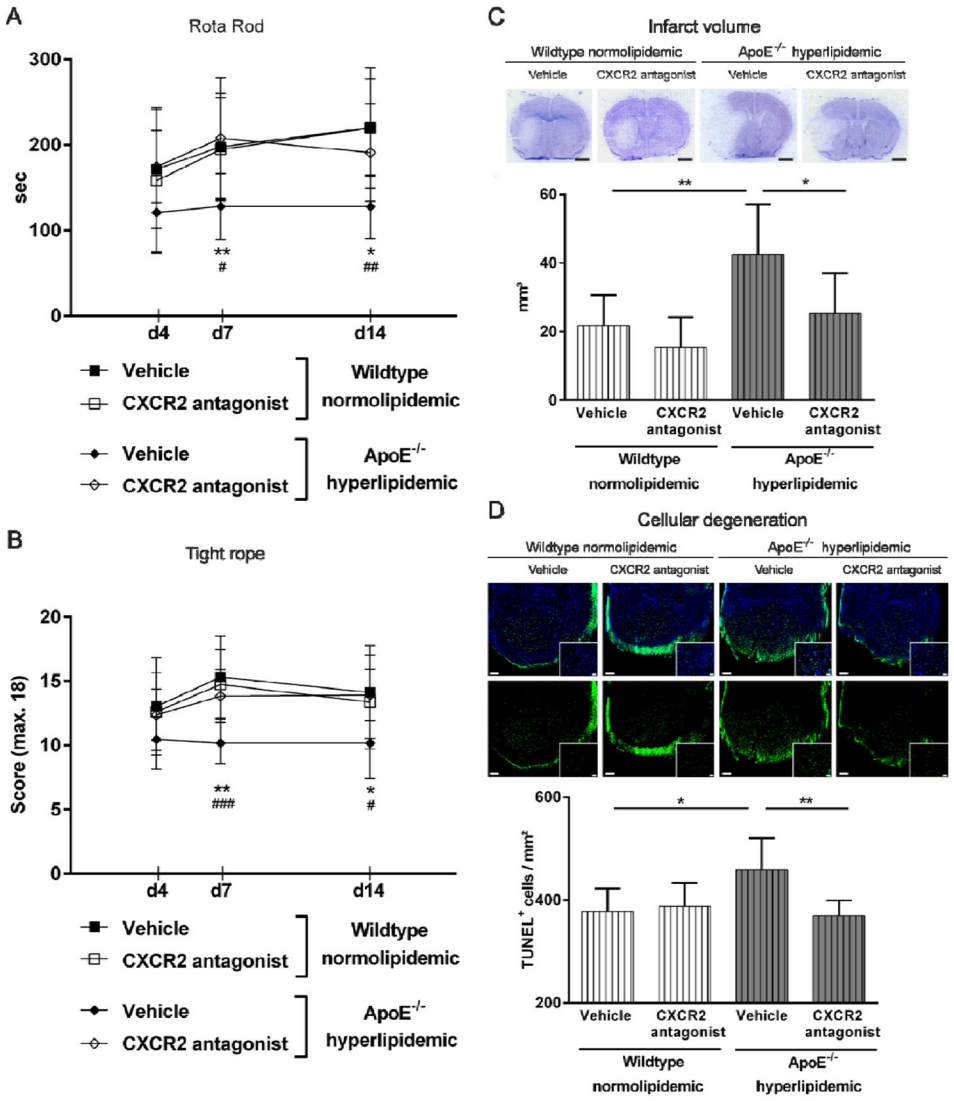

Fig. 1. The CXCR2 antagonist SB225002 promotes functional recovery and reduces brain injury in ischemic hyperlipidemic mice

The CXCR-2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) were i.p. injected at 0 hours, 24 hours and 48 hours post-ischemia in wildtype mice fed with normal chow (normolipidemic) or ApoE$^{-/-}$ mice fed with high cholesterol diet (hyperlipidemic). Post-stroke functional recovery was analyzed on days 4, 7 and 14 using the rota rod (A) and the tight rope (B) tests (n=11–12). Maximal testing time was 300 s for the rota rod test (A). The tight rope test (B) was analyzed using a validated score from 0 (min) to 18 (max). Infarct volumes (C) and cellular degeneration (D) were assessed 72 hours post stroke using cresyl violet staining (C, top) and TUNEL staining (D, top) (n=8–9). *p<0.05 and **p<0.01 ApoE$^{-/-}$ hyperlipidemic /vehicle vs. wildtype normolipidemic / vehicle; # p<0.05 and ## p<0.01 ApoE$^{-/-}$ hyperlipidemic /vehicle vs. ApoE$^{-/-}$ hyperlipidemic / CXCR2 antagonist. Scale bars: 1 mm in (C); 500 μm (large scale images) and 50 μm (insets) in (D).

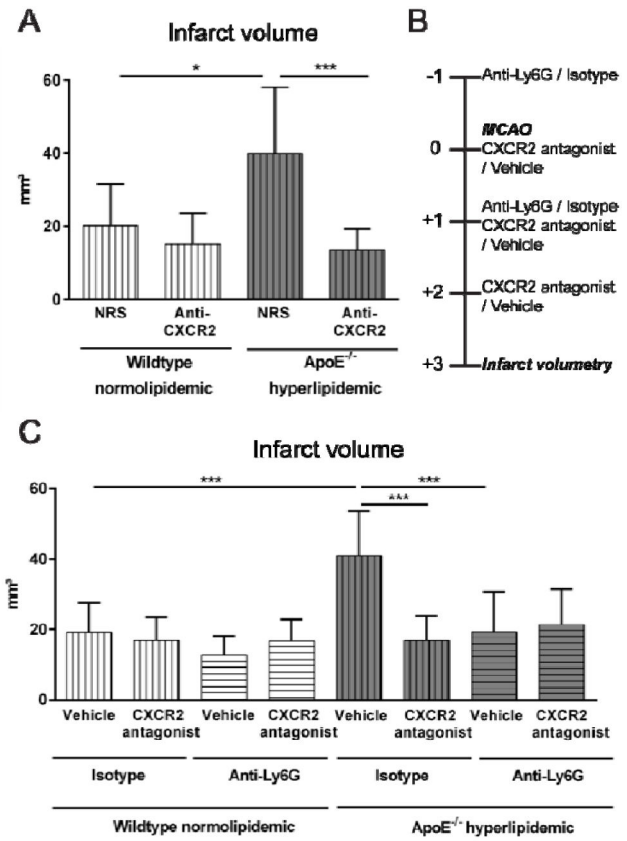

Fig. 2. Anti-CXCR2 treatment results in similar neuroprotection as SB225002 whose neuroprotective capacity is abrogated in neutrophil-depleted hyperlipidemic ApoE$^{-/-}$ mice

Infarct volumes were determined in ischemic wildtype mice fed with normal chow (normolipidemic) or ApoE$^{-/-}$ mice fed with high cholesterol diet (hyperlipidemic) which received either anti-CXCR2 serum or normal rabbit serum (NRS) at 0 hours, 24 hours and 48 hours post-ischemia (A) (n=8–9). For neutrophil depletion normolipidemic wildtype and hyperlipidemic ApoE$^{-/-}$ mice were treated with anti-Ly6G or isotype control antibody 24 hours prior to and 24 hours post-ischemia (B). The CXCR-2 inhibitor SB225002 (2 mg/kg, CXCR2 antagonist) or vehicle (1% DMSO in PBS) were i.p. injected at 0 hours, 24 hours and 48 hours post-ischemia (B, C) (n=7–8). Infarct volumes were determined at 72 hours post ischemia using cresyl violet staining (A–C). *p<0.05, ***p<0.001.

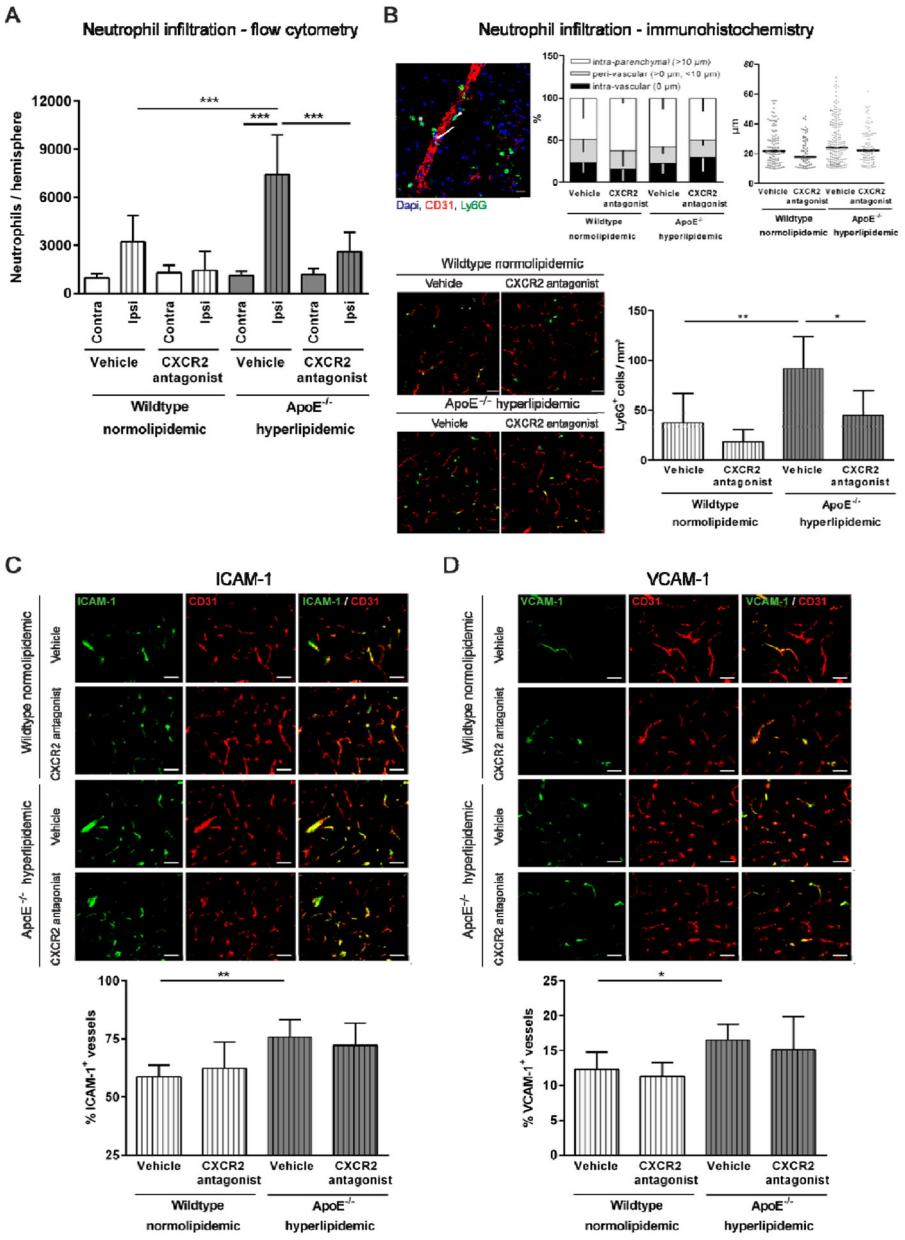

Fig. 3. CXCR2 antagonization reduces neutrophil infiltration without affecting endothelial activation

Wildtype mice fed a normal chow (normolipidemic) or ApoE−/− mice fed a Western diet for 6 weeks (hyperlipidemic) were exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke. Analysis was performed at 72 hours post-ischemia. The amount of brain infiltrated neutrophils was quantified in ipsi- and contralateral hemispheres using flow cytometry by gating of PI^{-}, $CD45^{high}$, SSC^{high}, $Ly6G^{+}$ cells (A, 3 individual experiments, 3–4 brains pooled per group and experiment). Local neutrophil accumulation and distribution was assessed via immunohistochemical stainings for Ly6G on ischemic brain tissue sections at the level of bregma (B, n=6). The exact localization of neutrophils was

determined through Ly6G/CD31 co-stainings which were evaluated by confocal microscopy (B, top left image). Distances between single neutrophils (Ly6G, green) to the most adjacent vessel (CD31, red) were measured to differentiate between intra-vascular (0 μm, arrow), peri-vascular (0–10 μm, arrowhead) and intra-parenchymal (>10 μm, asterisk) neutrophils. The percentage of neutrophils within the indicated regions was calculated (B, top middle panel) and distances for intra-parenchymal cells were measured (B, top right panel). Finally, the total amount of neutrophils was quantified (B, bottom). ICAM-1 (C) and VCAM-1 (D) stainings combined with stainings for the pan-endothelial cell marker CD31 were performed on ischemic brain tissue sections. Positive ICAM-1, CD31 and VCAM-1 vessels were counted and the percentage of ICAM-1 (C) and VCAM-1 (D) positive vessels was calculated (n=6). Scale bars: 50 μm (C, D, A bottom) and 25 μm (A top). *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

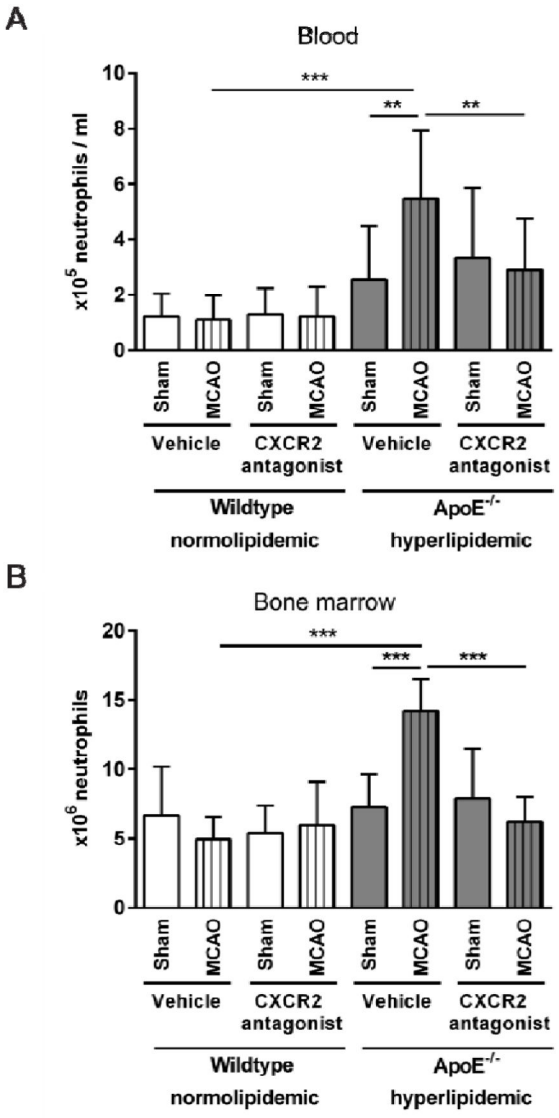

Fig. 4. Stroke-induced peripheral neutrophilia in hyperlipidemic mice is decreased by CXCR2 antagonization

White blood cells isolated from whole blood and bone marrow cells isolated from left and right femurs and tibiae were analyzed by flow cytometry after erythrocyte lysis. Absolute neutrophil counts were determined by quantification of PI^{-}, $CD45^{+}$, $Ly6G^{+}$, SSC^{high} cells in isolated blood (A) and bone marrow (B) cells of normolipidemic wildtype mice and hyperlipidemic $ApoE^{-/-}$ mice that were either sham operated or exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke (n=9–12). **p<0.01, ***p<0.001.

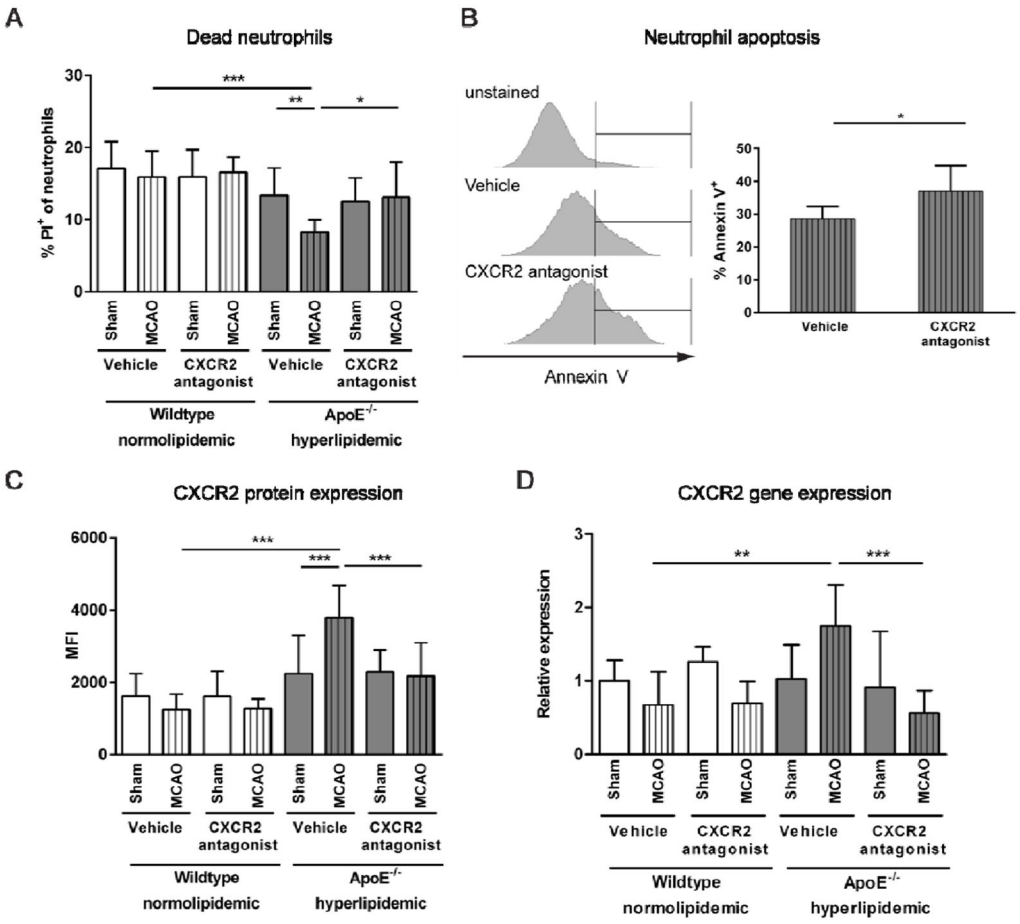

Fig. 5. The CXCR2 antagonist SB225002 increases peripheral neutrophil apoptosis and reduces CXCR2 expression in ischemic hyperlipidemic mice

Dead neutrophils were determined by quantification of propidium iodide (PI) positive cells of $CD45^+$, $Ly6G^+$, SSC^{high} cells via flow cytometry of isolated bone marrow cells in wildtype mice on normal diet or $ApoE^{-/-}$ mice on Western diet for 6 weeks that were either sham operated or exposed to tMCAO (A, n=9–12). Administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) was performed at 0 hours, 24 hours and 48 hours post stroke. To verify analysis of PI stainings, additional stainings for Annexin V positive neutrophils in the bone marrow of ischemic hyperlipidemic $ApoE^{-/-}$ mice comparing effects of vehicle and the CXCR2 antagonist SB225002 were performed (B, n=6). Representative histograms of $CD45^+$, $Ly6G^+$, SSC^{high} cells showing AnnexinV staining intensities (left) and quantification of the proportion of apoptotic neutrophils are shown (B). CXCR2 expression was quantified on bone marrow neutrophils via flow cytometry analyzing mean fluorescence intensity (MFI, C, n=9–12) and by gene expression analysis of CXCR2 via real time PCR on MACS-sorted bone marrow neutrophils (D, n=4–6). Mean values of ΔΔct values (relative expression) are presented in D. *p<0.05, **p<0.01, ***p<0.001.

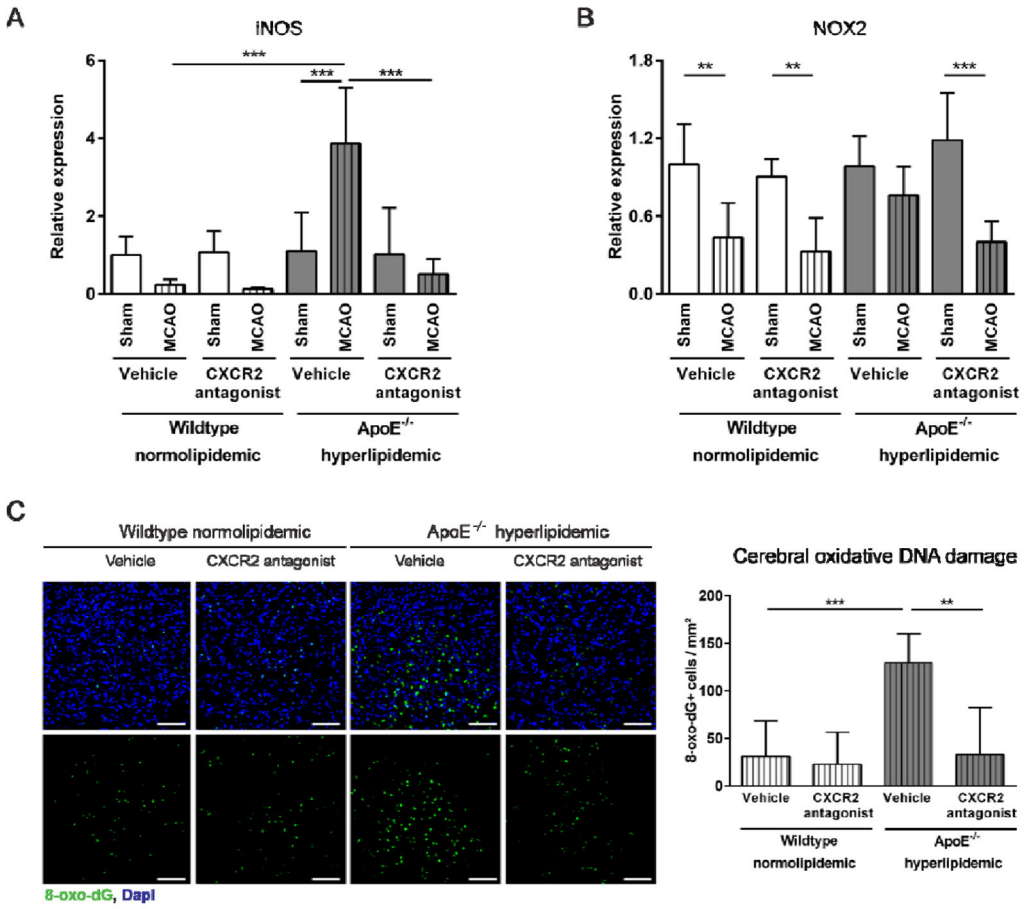

Fig. 6. Expression of oxidative stress-related enzymes in bone marrow neutrophils and cerebral oxidative DNA damage of ischemic hyperlipidemic mice are modulated by CXCR2 antagonization

mRNA expression analysis of inducible nitric oxide synthase (iNOS) (A) and NADPH oxidase 2 (NOX2) (B) was analyzed in (MACS)-sorted $Ly6G^+$ bone marrow neutrophils at 72 hours post-ischemia of wildtype mice fed a normal chow or $ApoE^{-/-}$ mice fed a Western diet for 6 weeks that were either sham operated or exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke. Mean values of ΔΔct values are presented (relative expression, n=4–6). Oxidative DNA damage was analyzed by immunohistochemical detection and quantification of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) positive cells in ischemic brain tissue (n=4–6). Scale bar: 50 μm. **p<0.01, ***p<0.001.