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Abstract

Impaired skin wound healing is a major medical problem in diabetic subjects. Kinins exert a number of vascular and other actions limiting organ damage in ischemia or diabetes but their role in skin injury is unknown. We investigated, through pharmacological manipulation of kinin B1 and B2 receptors (B1R/B2R), the role of kinins in wound healing in non-diabetic and diabetic mice.

Using two mouse models of diabetes (streptozotocin-induced and db/db mice) and non-diabetic mice, we assessed effect of kinin receptor activation or inhibition by subtype selective pharmacological agonists (B1R and B2R) and antagonist (B2R) on healing of experimental skin wound. We also studied effects of agonists and antagonist on keratinocytes and fibroblasts *in vitro*.

B1 and B2 receptor mRNAs level increased one to two fold in healthy and wounded diabetic skin compared to non-diabetic skin. Diabetes delayed wound healing. B1R agonist had no effect on wound healing. In contrast, B2R agonist impaired wound repair in both non-diabetic and diabetic mice, inducing skin disorganization and epidermis thickening. *In vitro*, B2R activation unbalanced fibroblast/keratinocyte proliferation and increased keratinocyte migration. These effects were abolished by co-administration of B2R antagonist. Interestingly, in the two mouse models of diabetes B2R antagonist administered alone normalized wound healing. This effect was associated with induction of MCP1/TNFα mRNAs.

Thus, stimulation of kinin B2 receptor impairs skin wound healing in mice. B2R activation occurs in the diabetic skin delays wound healing. B2R blockade improves skin wound healing in diabetic mice and is a potential therapeutic approach to diabetic ulcers.

Abbreviations

ACE: Angiotensin I-converting enzyme
AT1R: Angiotensin II type 1 receptor
AT2R: angiotensin II type 2 receptor
AU: arbitrary units
B1R: bradykinin type-1 receptor
B2R: bradykinin type-2 receptor
B1R-ag: bradykinin type-1 receptor agonist
B2R-ag: bradykinin type-2 receptor agonist
B2R-ant: bradykinin type-2 receptor antagonist
Diab: diabetic
DMEM: Dulbecco's Modified Eagle Medium
ERK1/2: p44/42 MAP-Kinase
FBS: Foetal Bovine Serum
IL-6: Interleukine 6
KKS: Kallikrein-Kinin(s) System
MCP-1: Monocyte Chemoattractant Protein-1
NBCS: New Born Calf Serum
NonDiab: non-diabetic
PBS: Phosphate Buffered Saline
SAP: Serum amyloid P
STZ: streptozotocin
STZ-Diab: diabetes induced by streptozotocin
TGF-beta: Transforming growth factor beta
TNF-alpha: Tumor Necrosis Factor alpha

Introduction

Impaired skin wound healing is a source of morbidity, disability, loss of quality of life and mortality in subjects affected by diabetes. Foot ulcers affect at least once in their life about 15% of patients with diabetes. Amputation rate is high in affected patients[1-3]. The pathogenesis of chronic foot ulcers is multifactorial and still incompletely understood. Loss of insulin action, hyperglycaemia, ischemia secondary to micro- and macro-angiopathy, neuropathy and infection all contribute to delayed skin repair and favour tissue necrosis, increasing amputation risk[4]. Improving skin wound healing in patients with diabetes is an important therapeutic issue.

Skin healing after wounding involves several different biological processes and comprises an acute response phase with haemostasis and inflammation, a granulation phase with cell migration, angiogenesis, production of extracellular matrix, wound contraction and re-epithelisation and a late scar maturation phase. Various cell types are involved in these processes: platelets, inflammatory cells, endothelial cells, fibroblasts and keratinocytes[5]. In diabetes, all phases of wound healing are altered. Cellular infiltration, migration and proliferation, and granulation tissue formation are delayed, together with impaired collagen organization and reduced angiogenesis[5]. Moreover, chronic hyperglycaemia alters fibroblasts and keratinocytes phenotypes. While impairment of the different components of the healing process has been documented in diabetes, therapeutic options for improving wound healing in diabetic subjects remain currently limited.

The kallikrein-kinin system (KKS) is implicated in physiological vasodilatation[6,7], anticoagulation[8], inflammation[9], and angiogenesis[10]. Previous studies have documented the role of this system in limiting organ damage in the setting of hemodynamic, hyperglycaemic or ischemic aggression[7,11]. We hypothesized that KKS could play a role in cutaneous responses to injury, especially in diabetes. Indeed, kinins have several cellular targets involved in skin repair including platelets, endothelial cells, keratinocytes and fibroblasts. Studies have documented the presence of the different components of the KKS in healthy, traumatized or diseased human skin[12-16]. Kinin receptors, bradykinin type-1 receptor (B1R) and bradykinin type-2 receptor (B2R), have been shown to participate in hyperproliferative process in chronic skin inflammation model[17]. Kinins increase skin microvascular blood flow in rabbits and humans[18]. These studies suggest a role for KKS in skin physiology. However, involvement of KKS in skin repair in the setting of diabetes remains undocumented.

The aim of the present study was to investigate the role of kinins and their receptors in wound healing in diabetic mice, and to compare with non-diabetic animals. Effect of potent selective pharmacological B1R or B2R agonists and/or of B2R antagonist was studied *in vivo* in mice and *in vitro* in cultured fibroblasts and keratinocytes. The study shows that a) B2R stimulation has deleterious effects in the wounded skin, unbalancing fibroblast/keratinocyte proliferation and migration and delaying cicatrisation; b) B2R activation occurs in the diabetic skin and impairs wound healing; c) B2R blockade improves wound healing in diabetic mice.

Materials and methods

Animals, experimental protocols and treatments

Effects of B1R or B2R agonists or B2R antagonist on wound healing were studied, in both non-diabetic (NonDiab) and diabetic (Diab) mice. All mice were housed with a 12 h light/dark cycle and had free access to standard mice chow and water. All experimental procedures were approved by the local Ethics Committee for Animal Experiment Charles Darwin and performed in accordance with European legislation for the care and use of laboratory animals (L 358-86/609/EEC).

Murine model of type 1 diabetes Diabetes was induced in 10 weeks-old mice (C57BL/6J strain, male, Charles River Laboratories, France) by 5 daily i.p. injections of streptozotocin (STZ) (Sigma-Aldrich, France) (50 mg/kg body weight in 0.05 mol/L^{-1} sodium citrate, pH 4.5)[10,19]. Diabetes was confirmed by assessing fasting blood glucose level at 0, 7 and 30 days post STZ injection. After 5 weeks of confirmed diabetes (fasting blood glucose>300 mg/dl), excisional wounds were created on the back of the mice as described below. Control NonDiab animals were treated with vehicle only.

Murine model of type 2 diabetes Diabetic db/db mice were obtained from Janvier Labs (Saint-Berthevin, France). Experiments were performed on male mice, 12 week-old. Only mice with 4 weeks of fasting blood glucose>300 mg/dl were used.

Murine model of skin wound healing Animals were anesthetized by isoflurane inhalation (1.5% in O_2). A dorsal 8 mm diameter full-thickness wound was made on dorsal depilated and cleaned (povidone-iodine solution) skin using a sterile biopsy punch (Kai medical, Japan)[20,21]. Buprenorphine (0.05 mg/kg; Buprécare, Axience, France) was administered as analgesic agent ten minutes prior and 24 hours after biopsy. Wounds were harvested by taking high-resolution photos every 2-3 days and the area was quantified relative to a millimetre reference using Image Analyzer Software (ImageJ, NIH) and expressed as percentage of wound area measured at day 0. At day 11 after injury, corresponding to wound closure of NonDiab mice, animals were sacrificed by pentobarbital overdose (120 mg/kg) and a skin sample including wound and surrounding tissues was collected. One half of the sample was immediately fixed in 10% formalin for histological analysis, the other part being kept at -80°C for molecular analyses.

Treatments administration Immediately after wounding, mice were implanted i.p. with osmotic minipumps (Alzet, model 1002, Charles River, France) delivering the selective B1R agonist (SarLys[Hyp3,Igl5,DPhe8]desArg9-bradykinin, B1R-ag) or the selective B2R agonist ([Hyp(3),Thi(5),(N)Chg(7),Thi(8)]-bradykinin, B2R-ag)[22,23], both at non-hypotensive dose of 720 nmol/kg.day^{-1} (B1R-ag: 796 µg/kg.day^{-1}; B2R-ag: 813 µg/kg.day^{-1}) [10], associated or not with the specific B2R antagonist (HOE140, Icatibant, B2R-ant)[24] at a dose of 380 nmol/kg.day^{-1} (500 µg/kg.day^{-1}) [25]. B2R-ant was also administered alone at the same dosage. Control mice received only vehicle (Phosphate Buffered Saline, PBS). Treatments were pursued until sacrifice (n=7-30/group).

Histological analysis Skin biopsies fixed in 10% formalin and embedded in paraffin were cut into 7-µm section and stained with Masson's trichrome according to the manufacturer's instructions (Sigma-Aldrich, France). Photomicrographs were obtained using digital camera attached to light microscope (Leica DM 4000B and LAS v3.8 software). Epidermal thickness

was measured in wounded skin and healthy surroundings area by using Image Analyzer Software (ImageJ, NIH) (3 measurements for each compartment/section, 4 sections/animal, n=7-13/group). Structure and organisation of the granulation tissue and/or the scar were observed.

Wound closure and histological studies were performed blindly with regard to treatment.

Measurement of kinin receptors and cytokines mRNA level and circulating MCP-1 and SAP Real time PCR was performed to assess B1R and B2R mRNA levels. Total RNA was isolated from the skin, collected at day 11, using TRIzol (Invitrogen, France) and reverse transcribed with superscript II reverse transcriptase as previously described[26]. The cDNAs were amplified and quantified using TaqMan Universal Master Mix and Assays-on-Demand Gene Expression Probes for gene of B1R and B2R (Applied Biosystems, France) in an ABI PRISM-7000 Sequence Detection System (Applied Biosystems, France). Each sample was tested in triplicate. Data were normalized to 18S rRNA. Changes in the target gene were calculated by the $2^{-\Delta\Delta CT}$ comparative method for each sample[27]. Same protocol was used to determine levels of MCP-1, TNF-alpha, TGF-beta and IL-6, four major pro-inflammatory cytokines, in wounded skin, using probes from the same supplier. Plasma MCP-1 concentration was also determined in blood samples at 11 days using a mouse MCP-1 enzyme-linked immunosorbent assay kit (Invitrogen, France) according to the manufacturer's instructions. Serum Amyloid P (SAP), an inflammatory marker equivalent to C-reactive protein in man, was quantified at 11 days using a mouse Elisa test kit (Life Diagnosctic, UK).

Keratinocytes and fibroblasts cell culture

Keratinocytes (human keratinocyte cell line HaCaT; ATCC, Virginia) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Glucose concentration 4500 mg/l, Gibco, LifeTechnologies, UK) supplemented with 10% Foetal Bovine Serum (FBS, Gibco, LifeTechnologies, UK) and antibiotics (50 U/mL Penicillin, 50 U/mL Streptomycin, Gibco, LifeTechnologies, UK). Keratinocytes were used between passages 68 and 75.

Fibroblasts (NIH-3T3 mouse fibroblasts; Sigma-Aldrich, Missouri) were cultured in DMEM supplemented with 5% New Born Calf Serum (NBCS, Gibco, LifeTechnologies, UK), 5% FBS and antibiotics according to manufacturer's guidelines. Fibroblasts were used between passages 162 and 171.

Keratinocytes and fibroblasts were kept at 37°C in a 5% CO_2 environment. Culture media were changed every two-day. When the cells reached subconfluence, they were harvested using 0.05% trypsin–EDTA (Gibco, LifeTechnologies, UK), and fresh culture medium was added for obtaining single cell suspensions used for further study.

Cell proliferation assay Cell proliferation was measured using Quick Cell Proliferation Assay kit (Abcam, Cambridge, MA) according to the manufacturer's instructions[28]. This assay is based on cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Briefly, keratinocytes or fibroblasts were seeded on 96-well plates ($1x10^4$ cells/well) and incubated with two different concentrations of B1R-ag or B2R-ag ($1x10^{-6}$ mol.L^{-1} and $1x10^{-7}$ mol.L^{-1}) and/or with B2R-ant ($1x10^{-5}$ mol.L^{-1}) in DMEM supplemented with 2% FBS. Cell proliferation was measured after 24, 48, 72 and 96 hours of incubation. Optical density was measured at 440 nm with a reference wavelength of 650 nm using a precision microplate reader (iMark Microplate Absorbance Reader, BIORAD).

Results were expressed as percentage of control (DMEM supplemented with 2% FBS). Each experiment was performed in duplicate per condition and repeated three times.

Wound closure assay Spreading and migration capabilities of cells were assessed using a scratch assay[29]. Keratinocytes or fibroblasts were seeded on 6-well plates ($3x10^5$ cells/well). After reaching confluence, cultures were scratch-wounded with a 0.2 ml sterile pipette tip at the centre of each well and cell debris were removed by PBS washing. For keratinocytes, plates were pre-coated with collagen I (BD Biosciences, California). B1R-ag or B2R-ag ($1x10^{-6}$ $mol.L^{-1}$ and $1x10^{-7}$ $mol.L^{-1}$) and/or B2R-ant ($1x10^{-5}$ $mol.L^{-1}$) were added in DMEM supplemented with 2% FBS. Mitomycin C (Sigma-Aldrich, Missouri) (10µg/mL) was also added in medium to inhibit cellular proliferation. After 15 hours, photographs of the scratch width were taken every 3 hours until closure and scratch closure was quantified by monitoring the front of migration. Results were expressed in percentage of scratch width measured at hour 0. Each experiment was realized in duplicate per condition and repeated four times.

Western blotting Cells were deprived in FBS during 24 hours and then treated 0, 2, 5, 15 and 40 minutes with B2R-ag ($1x10^{-6}$ $mol.L^{-1}$) and/or B2R-ant ($1x10^{-5}$ $mol.L^{-1}$). Proteins were extracted from treated-cells using standard protein extraction protocols and were dosed using BCA protein assay (Pierce Protein Biology Products, USA). Fifteen-µg proteins were separated on a Mini-PROTEAN TGX gel (Biorad, USA) and membranes were blotted with phospho- and total p44/42 MAPK (Erk-1/2) (Thr202/Tyr204) rabbit antibody (dilution 1/1000; Cell signalling technologies, Danvers, USA). They were exposed to ECL plus western blotting reagents (Biorad, USA) and reactive bands were detected in ImageQuant LAS 4000 (GE Healthcare, France) and quantified using Multi Gauge software 2.0 (FujiFilm, Japan). Each blot was then stripped and re-probed with anti-β-actin antibodies for data normalization. Results were confirmed by repeating the experiment 3–5 times.

Statistical analysis

Data are presented as mean±SEM. Statistical analysis was performed using one-way or two-way ANOVA for comparing effect of diabetes and/or treatments in mice. One-way ANOVA was used for comparing effect of treatment on cell migration or proliferation and effect of diabetes on receptor mRNA level. ANOVA was followed by ad-hoc multiple comparison tests. Statistical significance was accepted at $p<0.05$.

Results

Treatments did not affect metabolic parameters. Body weight and plasma glucose level of the different groups of mice at the beginning and end of the experiment are presented in Table1. Treatment with B1R-ag, B2R-ag and/or B2R-ant did not affect these parameters (Table 1).

B1R and B2R mRNA levels are increased in diabetic skin. Analysis of bradykinin receptor levels in healthy skin and wounded area at day 11 demonstrated that B1R mRNA level were increased roughly 3 times in STZ-Diab mice compared to NonDiab mice ($p<0.01$). Similarly, B2R mRNA level were also increased 2 times compared to Non-Diab mice ($p<0.01$) (Fig.1).

B1R agonist treatment had no effect on wound closure. B1R-ag treatment had no effect on wound closure in NonDiab mice (Fig.2A) and in STZ-Diab mice (Fig.2B). Moreover, histological analysis showed that B1R activation did not alter skin organisation and had no effect on epidermal thickness, neither in intact surrounding skin nor in wounded skin (data not shown).

B2R activation delays wound closure in mice. In NonDiab mice, B2R-ag treatment significantly delayed wound healing from the 1st day of treatment onward (Fig.2A). Co-treatment with the B2R-ant totally abrogated the wound healing delaying effect of B2R-ag. Kinetic profile of wound closure in mice co-treated with B2R-ag and B2R-ant was indistinguishable from PBS-treated mice (Fig.2A). In STZ-Diab mice, wound closure was significantly delayed when compared with NonDiab mice (Fig.2B). On day 11, wounds were healed in NonDiab mice whereas they remained open in STZ-Diab mice (12.7 ± 1.2 %, $p<0.05$). In STZ-Diab mice, B2R-ag treatment resulted in a further significant delay in wound healing from the 1st day of treatment onward (Fig.2B). On day 11, wound area was 2.3 times larger in B2R-ag-treated STZ-Diab mice compared to PBS-treated STZ-Diab mice ($p<0.05$).

B2R blockade improves skin wound healing in two models of diabetic mice. B2R-ant alone had no significant effect on wound healing in NonDiab mice (Fig.2A) but significantly improved skin wound healing in diabetic mice. In STZ-Diab mice, wound area was reduced on the 1st day of B2R-ant treatment and was indistinguishable from NonDiab mice during the 11 days follow-up period (Fig.2B). The beneficial effect of B2R-ant on wound healing was also observed in another diabetic model, db/db-Diab mice. In these mice, B2R-ant significantly reduced wound area during the 11th days of treatment (Fig.2C). On day 11, wound area was 3.2 times smaller in B2R-ant-treated db/db-Diab mice compared to PBS-treated db/db-Diab mice ($p<0.05$).

B2R activation induces epidermal thickening and skin layer disorganization in wounded skin. B2R activation induced no alteration in intact part of skin in NonDiab and STZ-Diab mice (data not shown). But, in the wound of NonDiab and Diab mice, B2R-ag treatment induced skin layer disorganisation, with a defect in skin and epidermal maturation, hypervascularisation and hypercellularisation of granulation tissue (Fig.3A). In B2R-ag-treated NonDiab mice, epidermal thickness of wound area was increased compared to PBS-treated mice (111.1±8.7 μm vs 69.9±5.7 μm; $p<0.05$). This effect was abrogated by co-treatment with B2R-ant (56.3±8.2 μm; $p<0.05$) (Fig.3B). In Diab mice, treatment with B2R-ag had no further effect on epidermal thickness (Fig.3C).

B2R antagonist treatment restores wounded skin histology in diabetic mice. After B2R-ant treatment alone, the epidermis of Diab-mice was histologically indistinguishable from NonDiab mice (Fig.3A). B2R-ant decreased by 45% the epidermal thickness of diabetic wounds (112.4±21.9 µm vs 206.3±21.6 µm; $p<0.05$) (Fig.3C).

B2R-ant treatment increases mRNA level of MCP-1 and TNF-alpha. IL-6 and TGF-beta mRNA levels were not influenced by B2R-ag or B2R-ant treatment (data not shown). MCP-1 and TNF-alpha mRNA levels were not influenced by B2R-ag treatment but B2R-ant treatment significantly increased both mRNAs in the wounded skin compared to PBS-treated Diab-mice (Fig.4). However, treatment had no effect on plasma concentration of MCP-1 (Fig.4). Concentration of SAP was below detection level in all groups (data not shown).

B2R activation unbalances keratinocyte and fibroblast proliferation in vitro. Treatment with B1R-ag or B2R-ag significantly increased keratinocyte proliferation, assayed using Quick Cell Proliferation Assay kit (+36% to 46% at 48h, $p<0.05$) (Fig.5A). Moreover, fibroblast proliferation was significantly decreased by the B2R-ag (-33% at 48h, $p<0.05$) but was not altered by the B1R-ag (Fig.5B). These B2R-ag effects on cell proliferation were abolished by co-treatment with B2R-ant (Fig.5). B2R-ant alone had no effect on cell proliferation.

B2R activation stimulates keratinocyte but not fibroblast migration. Figure 6 shows that keratinocyte migration, assessed using scratch assay, was significantly stimulated with B2R-ag during the 30 hours scratch closure process and independently of the dose used (wound closure +32% compared to medium alone after 24h, $p<0.05$). This effect was abolished in presence of the B2R-ant (Fig.6B). B2R-ant alone or B1R-ag had no effect on cell migration (Fig.6B). B2R-ag treatment had no effect on fibroblast migration (data not shown).

B2R activation induces ERK phosphorylation in keratinocytes. B2R-ag induced phosphorylation of ERK-1/2 in keratinocytes. Peak phosphorylation occurred 5 to 40 min after stimulation ($p<0.05$) and thereafter, the phosphorylation level gradually decreased (60-120 min). This effect was blocked by B2R-ant co-treatment (Fig.7).

Discussion

The main findings of the present study are that kinin B2R activation delays healing in non-diabetic and diabetic mouse skin wound models. Treatment with a selective B2R antagonist accelerates skin wound repair in diabetic mice. The healing effect of B2R antagonist was consistent in two different murine models of diabetes, close to either type 1 or type 2 human diabetes. The deleterious effects of B2R activity in the wounded skin may involve unbalancing of keratinocyte and fibroblast proliferation during the re-epithelialisation and scar remodelling phases after injury.

Previous reports indicated that B1R and B2R are present in healthy human skin and in skin of patients with cutaneous wound or proliferative skin diseases[12-14]. We show that both receptors are present in murine skin and are upregulated by diabetes mellitus.

Kinins can exert their biological actions through these two types of G protein-coupled membrane receptors. While the B2R is constitutively present in most tissues and mediates the main effect of kinins, the B1R is considered as being mainly inducible and its functions are less well understood. Interestingly, we recently showed that B1R activation protects the heart against ischemia damage[30] and has pro-angiogenic effects in a diabetic mouse model of hindlimb ischemia[10]. However in the skin, B1R activation had no effect on wound healing in mice, diabetic or non-diabetic. This receptor does not seem to be importantly involved in control of skin trophicity in diabetes, despite its strong induction by chronic hyperglycaemia.

By contrast, B2R activation disorganized the architecture of the wound, increased epidermis thickness and significantly delayed wound healing in mice. The deleterious effect of B2R activation on wound repair was observed in both non-diabetic and diabetic mice and was additive to the effect of diabetes. Co-treatment with a specific B2R antagonist abrogated the effects of B2R agonist administration indicating that these effects were truly due to B2R activation.

Wound healing involves tightly regulated humoral and cellular processes. Alteration of these timely-controlled processes by chronic hyperglycaemia expands tissue damage and delays tissue repair[31]. After a cutaneous injury, the healing process can be divided into four successive, overlapping phases: coagulation, inflammation, cell migration and proliferation, and remodelling. Kinins, through B2R activation, could likely influence several of these phases.

The first steps of wound healing are haemostasis and inflammation[31]. Kinins, through vascular endothelium activation, have anti-clotting and profibrinolytic effects[32,33], increase blood flow and trigger leucocyte migration[34]. In our study, pharmacological manipulation of B2R activity influenced wound repair already on the first day of follow-up after surgery. This observation suggests that kinins influence the initial phase of wound repair. The absence of effect of B2R agonist treatment on inflammatory markers in plasma or on cytokine gene expression in scar suggests that inflammation does not play an important role in B2R effect in wounded skin. On the other hand, B2R antagonist induced gene expression of MCP-1 and TNF-alpha, two cytokines previously shown to be involved in wound repair that may thus mediate, at least in part, the beneficial effect of B2R inhibition [35,36]. The mechanism of MCP-1 and TNF-alpha induction during B2R inhibition remains however undocumented.

Kinins can also influence late phases of healing. Our histological results show that B2R agonist induced skin disorganisation and increased epidermal thickness and hypercellular granulation tissue. Moreover, previous studies suggested that bradykinin enhances keratinocyte motility[37] and differentiation even if effect on cell proliferation is

unclear[13,15]. All these findings suggest that kinins influence proliferative and remodelling phases of healing. In this context, we studied *in vitro* effect of B2R activation on proliferation and migration of the two major cell types involved in scar formation: keratinocytes and fibroblasts. Our results show that B2R activation inhibited fibroblast proliferation but increased keratinocyte proliferation and migration in a cell layer injury model. Despite limitation in extrapolation of data from *in vitro* to *in vivo* settings due to use of established cell lines and different species, these effects of B2R agonist are consistent with the wound repair delaying effect observed in mice. Indeed, accumulation of fibroblasts, formation of granulation tissue, reconstitution of the dermis and reformation of an intact epidermis *via* keratinocyte migration and proliferation are important features of the wound healing process[31]. Skin layer disorganization, involving stratum corneum, epidermis and dermis, observed in mice treated with B2R agonist, especially in diabetic mice, may thus reflect unbalance between hypo-proliferation of fibroblasts and hyper-proliferation of keratinocytes, impairing healing. Stimulation of keratinocyte proliferation and migration *in vitro* by B2R activation is in line with the thickening of epidermis observed in B2R agonist treated mice.

B2R activation effects on keratinocytes were associated with ERK-1/2 phosphorylation. This result is in agreement with previous observations showing that keratinocyte migration, proliferation and wound re-epithelialization involve ERK activation[38-40] and confirms the importance of this pathway in keratinocyte functionality.

The deleterious effect of B2R stimulation on wound healing was additive to the effect of diabetes. Abnormal keratinocyte and fibroblast migration, proliferation and differentiation, and decreased vascularisation contribute to delayed wound healing in diabetic patients[4,41]. In these patients, keratinocytes in epidermis of chronic ulcers are highly proliferative[42]. B2R agonist treatment probably worsened keratinocyte hyper-proliferation due to diabetes and stimulated migration, resulting in abnormal scar remodelling.

Interestingly, whereas B2R antagonist alone had no effect on the wounded skin in non-diabetic mice, it significantly accelerated wound repair in diabetic mice. Indeed, B2R blockade restored the normal proliferation and maturation pattern of the wounded epidermis in a murine model of type 1 diabetes (STZ). The beneficial effect on wound healing of B2R antagonist was extended to a second diabetic mouse model, closer to type 2 human diabetes (db/db mice). These observations indicate that endogenous kinins exert deleterious effects opposing wound repair in diabetic skin. The lack of effect of B2R antagonist in non-diabetic mice may be explained by low kinins bioavailability and/or reduced B2R synthesis in the skin in normoglycaemic condition. By contrast, in diabetes, B2R synthesis is upregulated in the skin and kinins production may also be enhanced.

The study highlights the organ specificity and duality of action of B2R. B2R activation simultaneously aggravates tissue damage in the wounded skin and reduces ischemic or hyperglycaemic tissue damage in heart and kidney[11,26,43]. Kinins are mainly inactivated in the circulation by the angiotensin I-converting enzyme (ACE/kininase II). ACE inhibitors increase kinin biodisponibility and are largely used clinically in a number of cardiovascular and renal indications, including in diabetes. No side effect related to impairment of skin wound healing or aggravation of diabetic ulcers has however been reported in clinical trials with ACE inhibitors. ACE inhibitors have not been studied experimentally in wound healing but angiotensin II type 1 receptor (AT1R) blockers and AT1R gene inactivated mice have been shown to display delayed skin wound healing in both non-diabetic and diabetic conditions[44]. As AT1R inhibition triggers angiotensin II type 2 receptor (AT2R) stimulation and subsequent kinin formation and B2R activation[45,46], kinins might be

involved in the deleterious effect of AT1R blockade observed experimentally in the skin, although this remains speculative. Like for ACE inhibitors no side effect related to skin healing has been observed in clinical trials with AT1R blockers. In our study, the absence of effect of B2R antagonist in non-diabetic mice may be explained by low kinins bioavailability and/or reduced B2R synthesis in the skin in normoglycaemic condition. By contrast, in diabetes B2R synthesis is upregulated in the skin and kinin production may also be enhanced. Kinins are produced by tissue kallikrein and/or activated plasma prekallikrein. Tissue kallikrein is present in the skin[12,14,15]. However, in tissue kallikrein deficient mice[7,47], either rendered diabetic or not, we did not observe any alteration in wound repair, suggesting that tissue kallikrein is not importantly involved in kinin production in the wounded skin (unpublished results). On the other hand, plasma prekallikrein increases in diabetes[48] and this enzyme might be responsible for kinins production in the wounded skin, although this remains speculative.

In conclusion, B2R but not B1R activation exerts deleterious effects in the wounded mouse skin resulting in delayed healing, especially in diabetic animals. B1R and B2R are upregulated by diabetes in skin. Treatment with a B2R antagonist accelerates wound repair in two different mouse models of diabetes. Improving skin healing is still a therapeutic issue in diabetic patients, especially for treatment of foot ulcers. These results indicate that investigation of kinin B2 antagonism in human diabetic wound healing is warranted, and that kinin B2R antagonism could become a new treatment for cutaneous injury. It is worth noting that icatibant, a first generation pseudopeptide kinin B2R antagonist synthesized decades ago[24], has recently been approved for clinical use in an unrelated indication, angioedema [49].

Clinical perspectives

- Impairment of skin wound repair is a major complication of diabetes, favouring development of foot ulcers and contributing to their poor prognosis. Improving skin healing is a therapeutic issue in diabetic patients.
- Our study revealed that treatment with a kinin B2-receptor antagonist normalizes wound repair in two mouse models of diabetes, close to either type 1 or type 2 human diabetes.
- B2-receptor antagonism, already approved clinically in an unrelated indication, may become new treatment for cutaneous injury in diabetic patients.

Author contributions

D.D., L.W, V.D., F.A-G., R.R., N.B. made substantial contribution to the design, acquisition and analysis of data and have drafted the manuscript. C.C., C.T. made substantial contribution to acquisition of data. All authors have reviewed and approved the final version.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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TABLE 1: Weight and blood glucose level of different experimental groups at time of wounding (beginning of the experiment) and 11 days after wounding (end of the experiment).

		Beginning of experiment		**End of experiment**	
	n	Weight (g)	Blood glucose (mg/dL)	Weight (g)	Blood glucose (mg/dL)
NonDiab	30	26.2 ± 0.5	178 ± 6	25.7 ± 0.4	156 ± 12
STZ-Diab					
PBS-treated	22	23.3 ± 0.2	471 ± 17 **	22.8 ± 0.3	464 ± 13 **
B1R-ag-treated	21	23.5 ± 0.2	500 ± 17 **	23.9 ± 0.2	452 ± 21 **
B2R-ag-treated	20	22.5 ± 0.2	456 ± 26 **	23.3 ± 0.3	438 ± 32 **
B2R-ant-treated	7	23.0 ± 0.3	460 ± 34 **	24.0 ± 0.5	453 ± 26 **
db/db mice					
PBS-treated	10	43.2 ± 0.9	386 ± 25 **	44.5 ± 1.2	379 ± 27 **
B2R-ant-treated	10	43.5 ± 0.4	385 ± 34 **	45.8 ± 0.8	394 ± 29 **

** $p<0.001$: glucose level in all Diab groups vs NonDiab. NS among Diab groups.

Figure legends

Fig.1: B1R and B2R mRNA levels are increased in diabetic skin.
Kinin receptor mRNA level in non-diabetic and diabetic mouse skin (wounded or not), measured by RT-qPCR. Data were normalized to 18S rRNA. Data are mean±SEM, n=7/group. **:$p<0.01$ Diab vs NonDiab

Fig.2: B2R but not B1R activation influences wound healing in NonDiab mice and in Diab mice.
Representative photographs of wound area at day 0 and 11 and time course of wound closure (**A**: NonDiab mice; **B**: STZ-Diab mice; **C**: db/db-Diab mice). Results are expressed in percentage of initial wound area at day 0. Data are mean±SEM, n=7-30/group. *:$p<0.05$ and **:$p<0.01$ vs PBS-control in each series; $:$p<0.05$ and $$:$p<0.01$ Diab vs NonDiab.

Fig.3: B2R but not B1R activation increases epidermal thickness and worsens skin disorganization in wounded skin in NonDiab and STZ-Diab mice.
(**A**) Representative photographs of Massons's trichrome-stained sections of wounds at day 11 (2x; 10x; 25xmagnification) documenting change in the skin organization. Note thickness of epidermis and vascularisation and cellularisation of dermis. Bar = 1mm. Blue arrows show hypercellularisation, black arrows hypervascularisation, dashed blue arrow epidermal thickening and dashed black arrows skin layer disorganisation. (**B**) Quantification of epidermal thickness of the scar in NonDiab mice. (**C**) Quantification of epidermal thickness of the scar in STZ-Diab mice. Data are mean±SEM, n=7/group.

Fig.4: B2R-ant treatment increases mRNA level of MCP-1 and TNF-alpha.
(A) MCP-1 and TNF-alpha mRNA level in scar of STZ-Diab mice treated with the B2R-ag or B2R-ant, measured by RT-qPCR. Data were normalized to 18S rRNA. (B) MCP-1 protein level in plasma of these mice. Data are mean±SEM, n=5-6/group.

Fig.5: B2R activation unbalances keratinocytes/fibroblasts proliferation.
Cell proliferation after 48h of treatment with B1R-ag, B2R-ag and/or B2R-ant (**A**: keratinocytes; **B**: fibroblasts) at indicated doses. Data are mean±SEM, n=6 wells/group.

Fig.6: B2R but not B1R activation stimulates keratinocyte migration.
(**A**) Representative images of keratinocytes migration (10×magnification) at 0, 15 and 30h post scratch. Scratch area is delimited by black box. (**B**) Scratch closure after 24h of treatment. Data are mean±SEM and expressed as percentage of scratch width measured at 0h. Each data point represents n=8 wells.

Fig.7: B2R activation induces ERK phosphorylation in keratinocytes.
Phosphorylation of ERK-1/2 on keratinocyte lysates assessed by Western blotting after 0, 2, 5, 15 and 40 minutes of tretament with B2R-ag ($1x10^{-6}$ $mol.L^{-1}$) and/or B2R-ant ($1x10^{-5}$ $mol.L^{-1}$). Results are representative of 3-5 independent experiments. *:$p<0.05$ vs t=0min.

Summary statements

Kinin B2-receptor activation impairs skin wound healing in mice, in part through imbalance of keratinocyte/fibroblast proliferation. Such activation occurs in diabetes and contributes to delaying wound healing. B2-receptor blockade restores normal wound healing pattern in mouse models of diabetes.

Figure 1

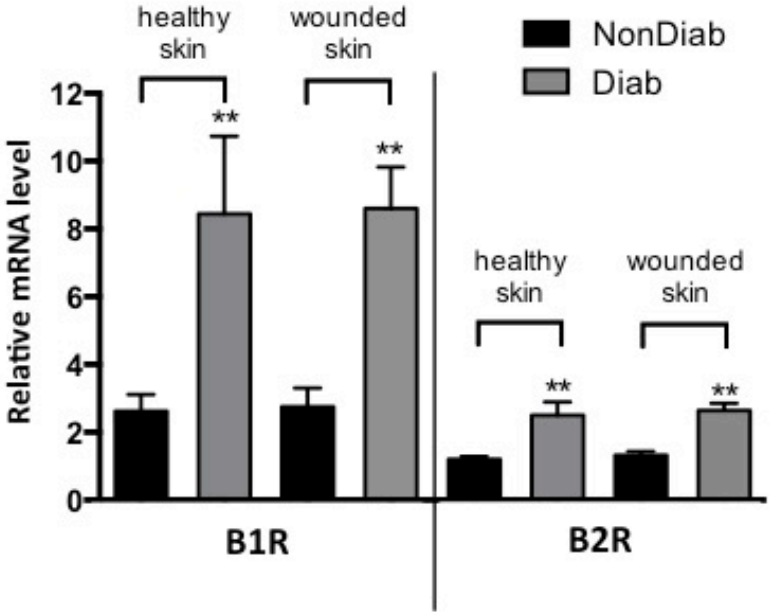

healthy skin
wounded skin
NonDiab
Diab
Relative mRNA level
12
10
8
6
4
2
0
**
**
healthy skin
wounded skin
**
**
B1R
B2R

Figure 2

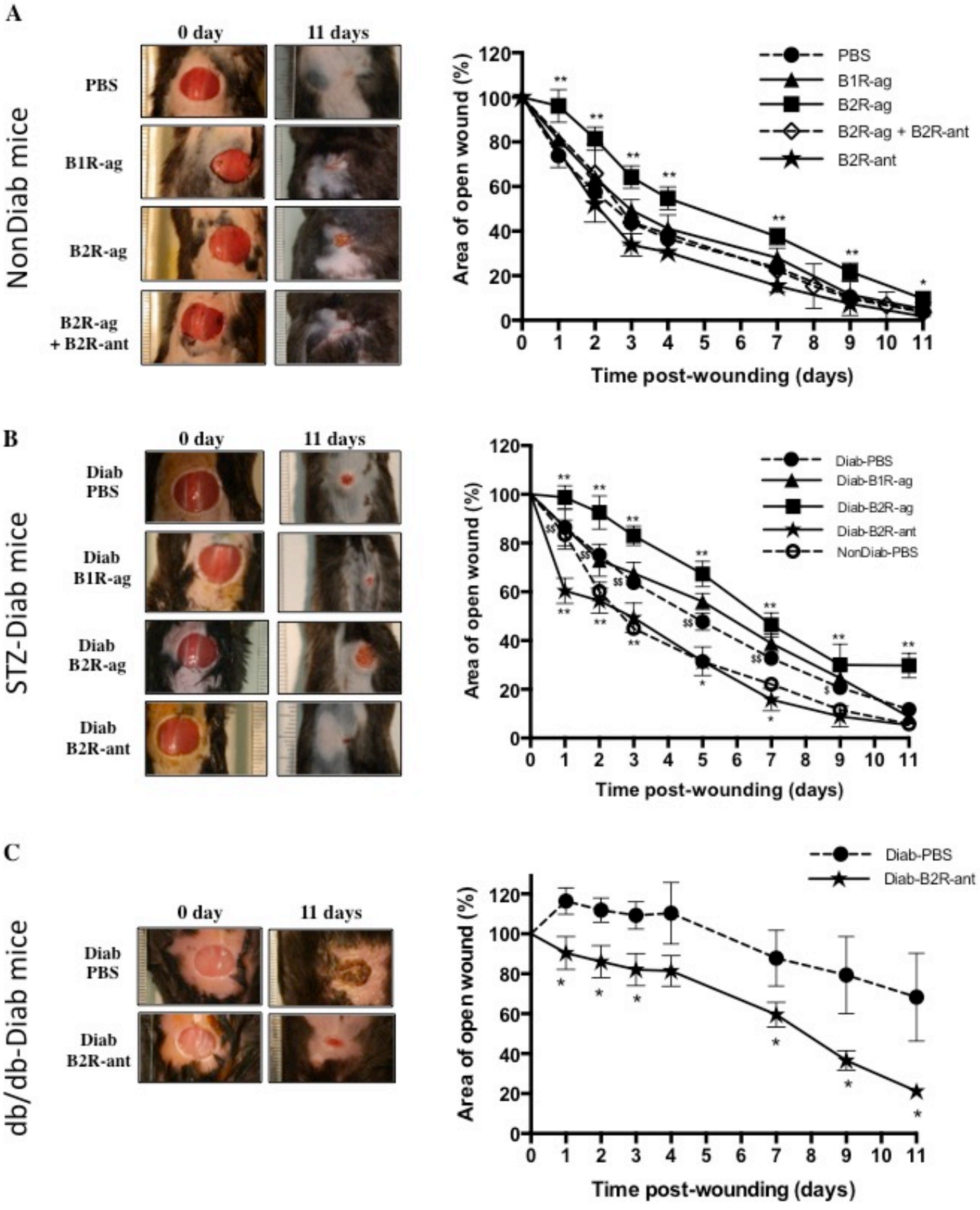

A
NonDiab mice
0 day
11 days
PBS
B1R-ag
B2R-ag
B2R-ag + B2R-ant
Area of open wound (%)
Time post-wounding (days)
PBS
B1R-ag
B2R-ag
B2R-ag + B2R-ant
B2R-ant
B
STZ-Diab mice
Diab PBS
Diab B1R-ag
Diab B2R-ag
Diab B2R-ant
Diab-PBS
Diab-B1R-ag
Diab-B2R-ag
Diab-B2R-ant
NonDiab-PBS
C
db/db-Diab mice
Diab-PBS
Diab-B2R-ant

Figure 3

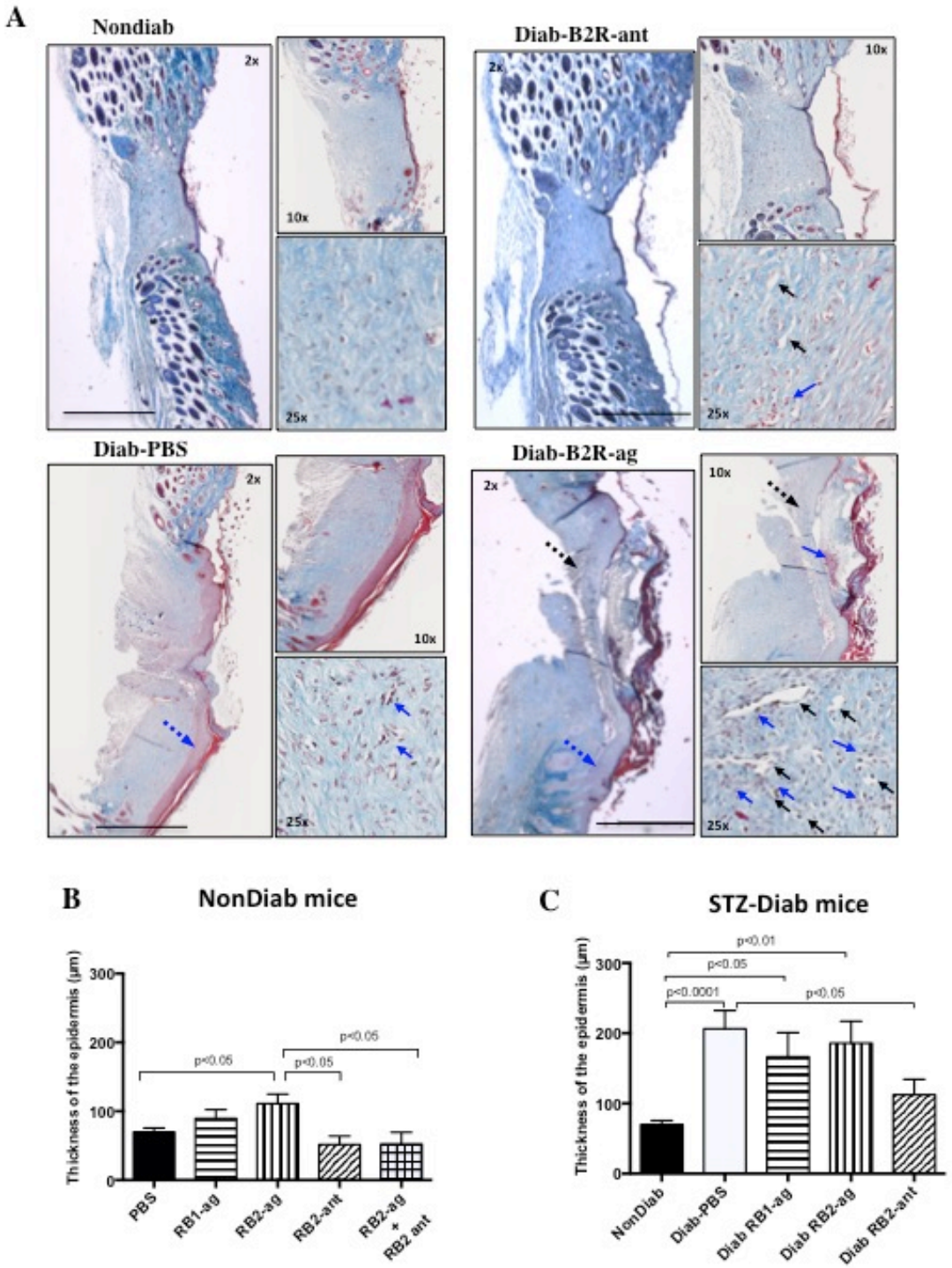

A
Nondiab
Diab-B2R-ant
Diab-PBS
Diab-B2R-ag
2x
10x
25x
B
NonDiab mice
Thickness of the epidermis (µm)
300
200
100
0
p<0.05
p<0.05
p<0.05
PBS
RB1-ag
RB2-ag
RB2-ant
RB2-ag + RB2 ant
C
STZ-Diab mice
Thickness of the epidermis (µm)
p<0.01
p<0.05
p<0.0001
p<0.05
NonDiab
Diab-PBS
Diab RB1-ag
Diab RB2-ag
Diab RB2-ant

Figure 4

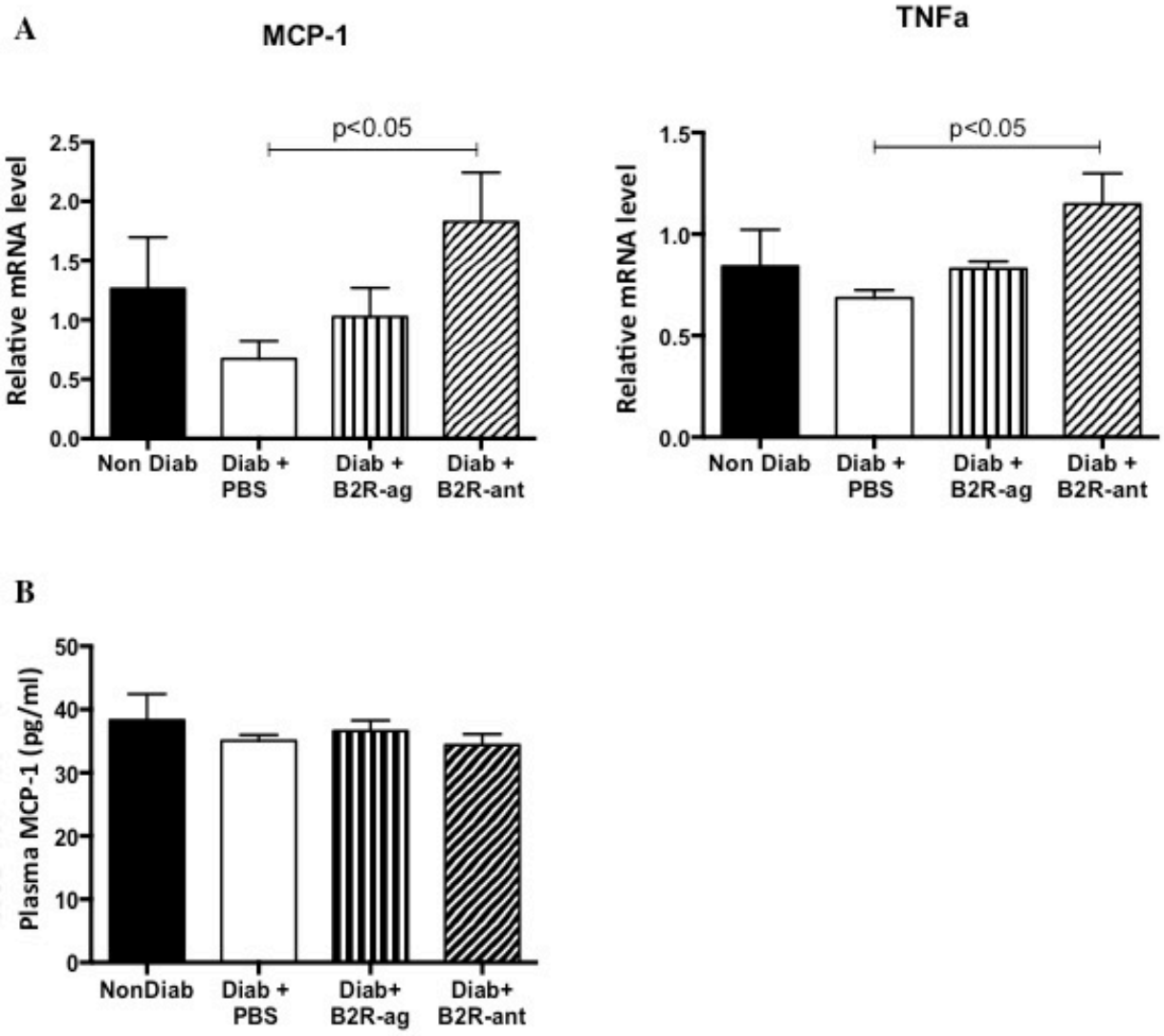

A
MCP-1
p<0.05
Relative mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
Non Diab
Diab + PBS
Diab + B2R-ag
Diab + B2R-ant
TNFa
p<0.05
Relative mRNA level
1.5
1.0
0.5
0.0
Non Diab
Diab + PBS
Diab + B2R-ag
Diab + B2R-ant
B
Plasma MCP-1 (pg/ml)
50
40
30
20
10
0
NonDiab
Diab + PBS
Diab+ B2R-ag
Diab+ B2R-ant

Figure 5

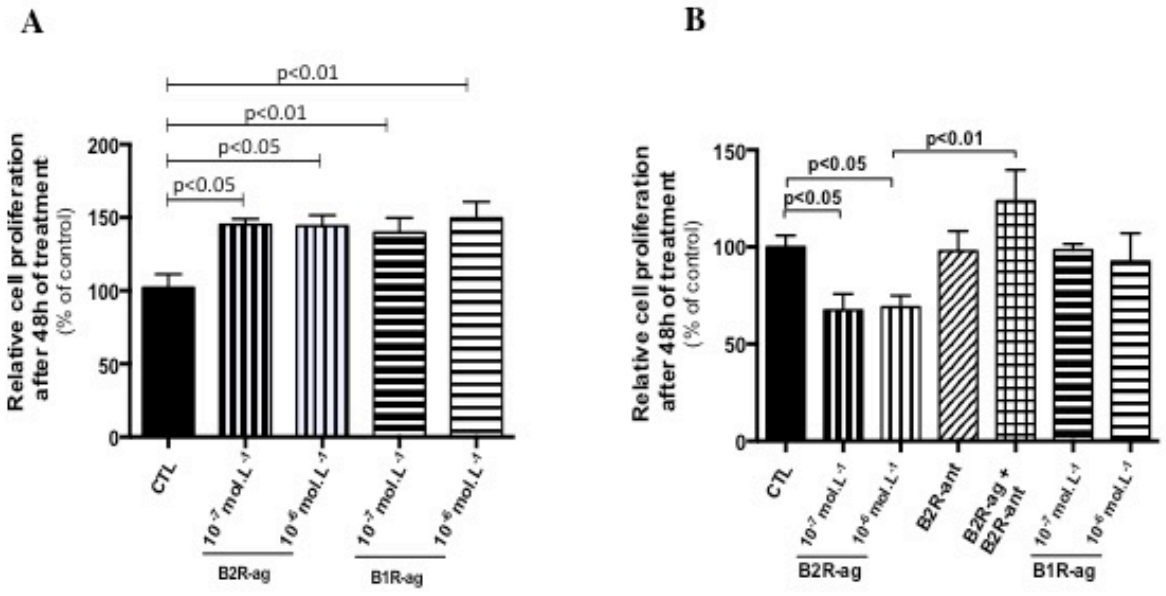

A
B
Relative cell proliferation after 48h of treatment (% of control)
p<0.01
p<0.01
p<0.05
p<0.05
200
150
100
50
0
CTL
10-7 mol.L-1
10-6 mol.L-1
10-7 mol.L-1
10-6 mol.L-1
B2R-ag
B1R-ag
p<0.05
p<0.05
p<0.01
150
100
50
0
CTL
10-7 mol.L-1
10-6 mol.L-1
B2R-ant
B2R-ag + B2R-ant
10-7 mol.L-1
10-6 mol.L-1
B2R-ag
B1R-ag

Figure 6

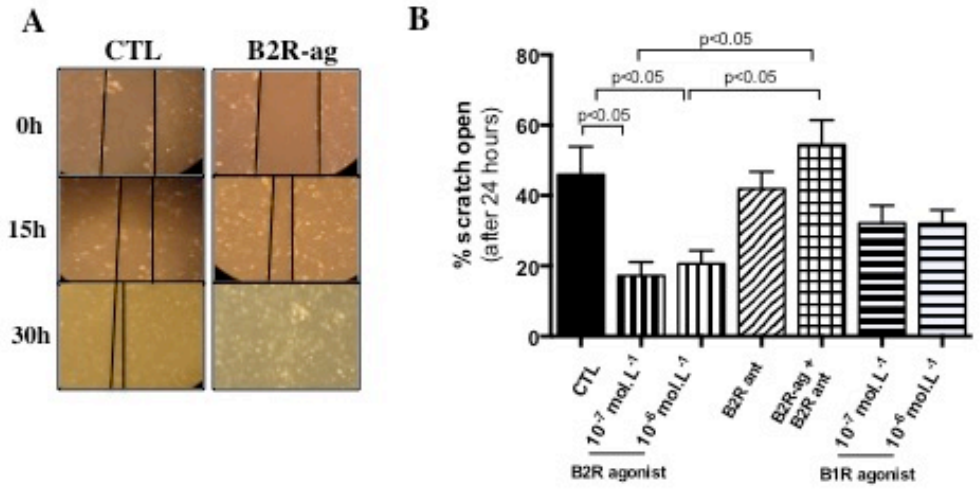
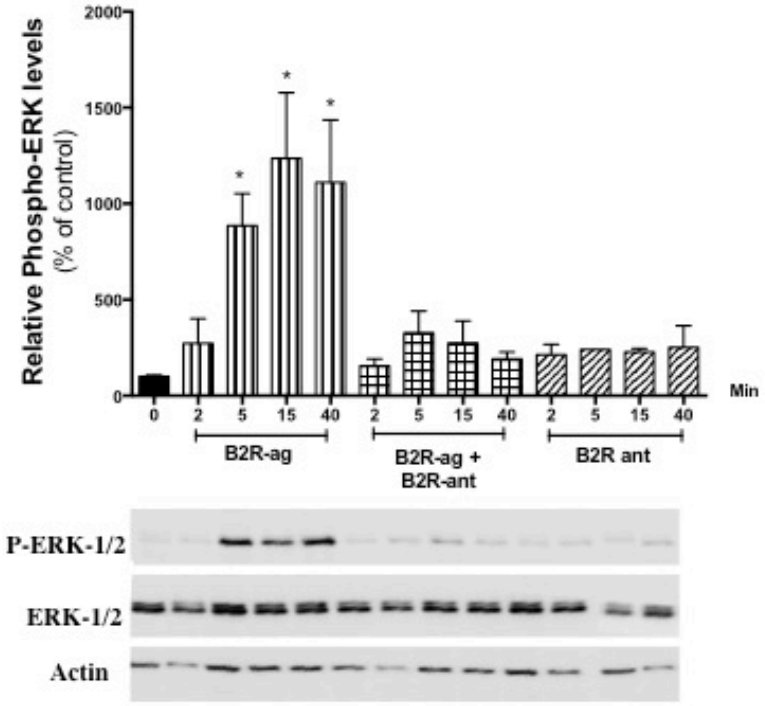

A
CTL
B2R-ag
0h
15h
30h
B
% scratch open
(after 24 hours)
80
60
40
20
0
p<0.05
p<0.05
p<0.05
p<0.05
CTL
10-7 mol.L-1
10-6 mol.L-1
B2R ant
B2R-ag + B2R ant
10-7 mol.L-1
10-6 mol.L-1
B2R agonist
B1R agonist

Figure 7


Relative Phospho-ERK levels
(% of control)
2000
1500
1000
500
0
*
*
*
0 2 5 15 40 2 5 15 40 2 5 15 40
Min
B2R-ag
B2R-ag +
B2R-ant
B2R ant
P-ERK-1/2
ERK-1/2
Actin