THE ROLE OF KININ B_1 RECEPTOR IN DIABETIC HYPERALGESIA

BY

BICHOY H. GABRA

A THESIS SUBMITTED IN PARTIAL FULFILLMENTS OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

DEPARTMENT OF PHARMACOLOGY
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LE RÔLE DU RÉCEPTEUR B_1 DES KININES DANS L'HYPERALGÉSIE DIABÉTIQUE

PAR

BICHOY H. GABRA

THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION DU GRADE DE PHILOSOPHIAE DOCTOR (Ph.D.)

DEPARTMENT DE PHARMACOLOGIE
FACULTÉ DE MÉDECINE
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To the memory of my father, Habachi
To the memory of my grandmother, Farida
To my mother, Salwa
To my aunt, Nagwa
To my sister, Miriam
To my niece, Jemma
To my nephew, Jacob
And very specially to my wife, Meriam and my son, Raphael

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SUMMARY

Autoimmune insulin-dependent type 1 diabetes involves an overproduction of cytokines, including interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which leads to T-cell-mediated pancreatic β-cell destruction. It is associated with a series of complications including nephropathy, retinopathy and painful diabetic neuropathy (PDN). Several neurovascular systems are activated in type 1 diabetes including the inducible bradykinin (BK) B_1 receptor (BKB_1-R) subtype which is generally absent or of little impact in healthy states, but highly induced or over-expressed under pathological conditions among which type 1 diabetes, where the over-production of cytokines, the hyperglycemia and the oxidative stress are critical factors for its up-regulation.

The present project aimed at studying the development of hyperalgesia in rodent models of type 1 diabetes and characterising the role of the inducible BKB_1-R in this diabetic complication, through the use of selective BKB_1-R agonists and antagonists. Nociception was evaluated with two types of thermal pain tests; spinal (tail immersion and tail flick tests) and supra-spinal (hot plate and plantar stimulation) tests.

Chemically streptozotocin (STZ)-induced diabetic mice and rats showed a marked hyperalgesia that was stable over 4 weeks following the STZ injection. Non-obese diabetic (NOD) and BioBreeding/Worcester (BB/Wor) rats, models of autoimmune spontaneous type 1 diabetes, similarly developed significant hyperalgesia over time (4-32 and 4-24 weeks of age, respectively). Hyperalgesia observed in NOD mice and BB/Wor rats did not correlate with hyperglycemia, but rather preceded the increase in the animal plasma glucose concentration. This finding supports the hypothesis that type 1 diabetic complications start early during the progression of the disease, even before the diagnosis of the state of hyperglycemia.

The selective BKB_1-R agonist, desArg^{9}BK (DBK), which did not affect nociception in control non-diabetic animals, potentiated hyperalgesia in STZ-diabetic and NOD mice. In addition, the acute and chronic administration of the selective BKB_1-R antagonists R-715 and R-954 significantly attenuated diabetic hyperalgesia in all tested animals models.

Our results also showed that stimulation of the inducible BKB_1-R activates several neurotransmitter systems responsible for the mediation of diabetic hyperalgesia including the substance P (SP), nitric oxide (NO) and calcitonin gene-related peptide (CGRP).

Nevertheless, the hyperalgesia observed in wild type diabetic mice was totally absent in the BKB_1-R-knockout (KO) diabetic mice in which DBK had no effect on nociceptive responses.

The profile of expression of the BKB_1-R in NOD mice was evaluated in several target organs and the results showed the induction of the BKB_1-R in the kidney and the spinal cord of diabetic NOD mice starting from 6 - 32 weeks of age.

In conclusion, the BKB_1-R subtype appears to mediate the manifestation of hyperalgesia in type 1 diabetic rodents. Thus, selective antagonism of this inducible receptor may constitute a novel and potential therapeutic approach for the treatment of PDN.

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RÉSUMÉ

Le diabète insulino-dépendent de type-1 est dû à une réponse auto-immune associée à la surproduction de cytokines, telles que l'interleukin-1β et le facteur de nécrose tumoral-α, qui mène à la destruction des cellules β des îlots pancréatiques. Cette maladie comporte une composante inflammatoire qui est responsable de nombreuses complications incluant la néphropathie, la rétinopathie et la neuropathie diabétique douloureuse. Plusieurs systèmes neurovasculaires sont activés dans le diabète de type 1 incluant le récepteur B_1 inductible de la bradykinine qui est généralement absent dans les conditions normales, mais induit dans plusieurs conditions pathologiques incluant le diabète de type 1, en raison de la surexpression des cytokines, de l'hyperglycémie et du stress oxydatif.

Dans le projet présent, on a évalué le développement de l'hyperalgésie dans plusieurs modèles de diabète de type 1 chez les rongeurs. De plus on a caractérisé le rôle du récepteur B_1 inductible de la bradykinine dans cette complication diabétique en utilisant des agonistes et des antagonistes sélectifs de ce récepteur. La nociception a été évaluée par deux types d'essais de douleur thermique : essais spinaux (l'immersion de la queue et le retrait de la queue) et essais supra-spinaux (la plaque chauffante, la stimulation plantaire).

Les souris et les rats traités à la streptozotocine ont démontré une hyperalgésie marquée qui était stable sur une période de quatre semaines après l'injection. Les souris non-obèse diabétique (NOD) et les rats BioBreeding/Worcester (BB/Wor), modèles de diabète de type 1 spontané, ont également développé un hyperalgésie significative et âge-dépendente (4-32 et 4-24 semaines d'âge, respectivement).

L'hyperalgésie observée chez les souris NOD et les rats BB/Wor n'était pas en correlation avec l'hyperglycémie, mais a plutôt précédé l'augmentation de la concentration plasmatique du glucose chez les animaux diabétiques. Ces résultats supportent l'hypothèse que les complications du diabète de type 1 débutent tôt durant la progression de la maladie, même avant la diagnostique de l'état d'hyperglycémie.

L'agoniste sélectif du récepteur B_1 de la bradykinine, la desArg^{9}bradykinine, n'a eu aucun effet sur la nociception chez les animaux témoins non-diabétiques. Cependant, cet agoniste a significativement potentié l'effet hyperalgésique chez les souris traitées à la streptozotocine et les souris NOD. De plus, le traitement aigu ou chronique avec les antagonistes sélectifs récepteur B_1 de la bradykinine, le R-715 et le R-954, a atténué d'une façon significative l'hyperalgésie diabétique chez tous les modèles testés.

Nos résultats ont aussi démontré que la stimulation du récepteur B_1 de la bradykinine active plusieurs systèmes neurotransmetteurs responsables de la médiation de l'hyperalgésie diabétique incluant la substance P, le monoxyde d'azote et le peptide relié au gène de la calcitonine.

Néanmoins, l'hyperalgésie observée chez les souris de type sauvage était complètement absente chez les souris déprivées du récepteur B_1 de la bradykinine chez lesquelles la desArg^{9}bradykinine n'a pas affecté la réponse nociceptive.

D'ailleurs, le profil d'expression du récepteur B_1 de la bradykinine chez les souris NOD a été évalué dans plusieurs tissus cibles et nos résultats ont démontré que l'induction du récepteur au niveau du rein et de la moelle épinière des souris NOD commence à 6 semaines jusqu'à 32 semaines d'âge.

Pour conclure, le récepteur B_1 inductible de la bradykinine semble être responsable de la manifestation de l'hyperalgésie chez les rongeurs diabétiques de type 1. Ainsi, l'antagonisme sélectif de ce récepteur suggère une avenue thérapeutique potentielle envers cette complication diabétique.

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AC	:	adenylate cyclase
ACE	:	angiotensin I converting enzyme
Ac-Lys	:	acetyl lysine
Ac-Orn	:	acetyl ornithine
AGEs	:	advanced glycosylation endproducts
ADP	:	adenosine diphosphate
AmM	:	aminopeptidase M
ARA	:	arachidonate
Arg	:	arginine
ARIs	:	aldose reductase inhibitors
ATPase	:	adenosine triphosphatase
BB/Wor	:	BioBreeding/Worcester
BB/Wor-DP	:	BioBreeding/Worcester-diabetic prone
BB/Wor-DR	:	BioBreeding/Worcester-diabetic resistant
BDNF	:	brain-derived neurotrophic factor
BK	:	bradykinin
BKB_1-R	:	bradykinin B_1 receptor
BKB_2-R	:	bradykinin B_2 receptor
bp	:	base pairs
Ca^{2+}	:	calcium
cAMP	:	cyclic adenosine monophosphate

cDNA	:	complementary deoxyribonucleic acid
cGMP	:	cyclic guanosine monophosphate
CGRP	:	calcitonin gene-related peptide
CNS	:	central nervous system
CNTF	:	ciliary neurotrophic factor
CPM	:	carboxypeptidase M
CPN	:	carboxypeptidase N
CTGF	:	connective tissue growth factor
DAB	:	3,3'diaminobenzidine
DAG	:	diacylglycerol
db/db	:	obese diabetic
DBK	:	desArg^{9}BK
DNA	:	deoxyribonucleic acid
DOCA	:	deoxycorticosterone acetate
eNOS	:	endothelial nitroc oxide synthase
GC	:	guanylate cyclase
GDNF	:	glial-cell-line-derived neurotrophic factor
GFAP	:	glial fibrillary acid protein
Gly	:	glycine
GPCR	:	G-protein-coupled receptors
h	:	hour
HMWK	:	high molecular weight kallikrein
IC2	:	intracellular loop 2

I/D	:	insertion/deletion
IGFs		insulin-like growth factors
Ile	:	isoleucine
IL-1β	:	interleukin-1β
i.p.	:	intraperitoneal
IP	:	prostanoid PGI_2 receptor
IP3	:	inositol 1,4,5-triphosphate
K^+	:	potassium
kb	:	unit of length for DNA fragments equal to 1000 nucleotides
KD	:	kallidin
kDa	:	kiloDalton; one kiloDalton (kDa) is equal to approximately the weight of one thousand hydrogen atoms, and is equivalent to 1.66×10^{-21} grams
KKS	:	kallikrein-kinin system
KO	:	knockout
LPS	:	lipopolysaccharide
LMWK	:	low molecular weight kallikrein
Lys	:	lysine
MAP-kinase	:	mitogen-activated protein kinase
MBP	:	myelin basic protein
MHC	:	major histocompatibilty complex
min	:	minute
mmol/l	:	millimole per liter
µm	:	micrometer

MOE	:	molecular operating environment
mRNA	:	messenger ribonucleic acid
Na^+	:	sodium
NAD^+	:	nicotinamide adenine dinucleotide
Nal	:	naphthylalanine
NGF	:	nerve growth factor
NEP	:	neutral endopeptidase
NF-κB	:	nuclear transcriptional factor kappa B
NKA		neurokinin A
NMDA	:	*N*-methyl-D-aspartste
NO	:	nitric oxide
NOD	:	non-obese diabetic
NOS	:	nitric oxide synthase
NS	:	non-specific binding
NT-3	:	neurotrophin-3
NT-4/5	:	neurotrophin-4/5
Oic	:	L-octahydroindole-2-carboxylic acid
PARP	:	poly(ADP-ribose) polymerase
PDN	:	painful diabetic neuropathy
PGI_2	:	prostacyclin I_2
Phe	:	phenylalanine
PI	:	phosphatidyl inositol
PLA_2	:	phospholipase A_2

PLC : phospholipase C

PKC : protein kinase C

PLD : phospholipase D

PRE : positive regulatory element

PTK : protein tyrosine kinase

Pro : proline

p53 : a gene that codes for a protein that regulates the cell cycle and hence functions as a tumor suppressor

RAGE : receptor of advanced glycation endproducts

s.c. : subcutaneous

sec : second

Ser : serine

SP : substance P

STZ : streptozotocin

TATA : a DNA sequence *(cis-element)* found in the promoter region of most genes (it is considered to be a promoter sequence).

TGF-β : tumour growth factor-β

TNF-α : tumour necrosis factor-α

TM : transmembrane

TRP : transient receptor potential

VWF : von Willebrand factor

INTRODUCTION

1. The kallikrein-kinin system

The kallikrein-kinin system (KKS) is an intricate endogenous system comprising the enzymes kallikreins, the protein precursors kininogens and the vasoactive peptides kinins (ROCHA E SILVA, *et al.*, 1949; ANDRADE & ROCHA E SILVA, *et al.*, 1956; ELLIOT *et al.*, 1959; Boissonnas *et al.*, 1960; REGOLI & BARABÉ, 1980; BHOOLA *et al.*, 1992; PESQUERO & BADER, 1998). Kinins are known to be important mediators of a variety of biological effects including cardiovascular homeostasis, inflammation and nociception (BHOOLA *et al.*, 1992; MARCEAU & BACHVAROV, 1998). They are probably the first mediators released in injured tissues from kininogens either by plasma kallikrein, which is activated early in the coagulation cascade, or tissue kallikrein, which is activated by proteases released at injured sites (BHOOLA *et al.*, 1992; WALKER *et al.*, 1995; PESQUERO *et al.*, 2000). Kinins are also known to be generated during noxious stimuli and some emerging evidence suggests that they are stored in neural elements of the central nervous system (CNS) where they are thought to play a role as neuromediators in various cerebral functions, particularly in the control of nociceptive information (BHOOLA *et al.*, 1992; COUTURE & LINDSEY, 2000).

1.1. Domain structure of kininogens

The low molecular weight kallikrein (LMWK) is a 66-kDa β-globulin with a plasma concentration of 160 mg/ml (2.4 mmol/l) and an isoelectric point of 4.7 (SCHMAIER *et al.*, 1986). The high molecular weight kallikrein (HMWK) is a 120-kDa α-globulin with a plasma concentration of 80 mg/ml (0.67 mmol/l) and an isoelectric point of 4.3 (SCHMAIER *et al.*, 1983; 1986). Human liver is a source for complementary deoxyribonucleic acid (cDNA)

for both kininogens (TAKAGAKI *et al.*, 1985; KITAMURA *et al.*, 1985) but human umbilical vein endothelial cells have been shown to contain HMWK messenger ribonucleic acid (mRNA) and to synthesize the protein (SCHMAIER *et al.*, 1988). Kininogen antigen also has been found in platelets, granulocytes, renal tubular cells, and skin (COLMAN & SCHMAIER, 1997).

The kininogens are proteins composed of multiple domains, each with associated activities (Figure 1). Binding of kininogen to its cell receptors facilitates bradykinin (BK) liberation in a circumscribed environment in which the peptide can bind to BK receptors and influence the local cellular milieu. Thus, one can view each function of the domains of the kininogens as participating in the whole protein kinin delivery activity. The kininogens, in general, can be divided into three portions: the heavy chain that is common to both HMWK and LMWK, the BK moiety, and the light chains that, as already stated, are unique to the HMWK and LMWK, respectively (Figure 1). Domains 1 through 3 comprise kininogens' heavy chain. Domain 4 is the BK region. Domain 5 for the LMWK ($D5_L$) is its unique 4-kDa light chain. Domains 5 and 6 of the HMWK ($D5_H$ or $D6_H$) are unique to this protein and comprise its light chain (COLMAN & SCHMAIER, 1997).

Little is known about the function of domain 1 except to note that it has a low-affinity binding calcium binding site whose role is unknown (HIGASHIYAMA *et al.*, 1987). Some evidence also indicates that a peptide from domain 1 inhibits atrial naturetic peptide (CROXATTO *et al.*, 1994).

Domains 2 and 3 contain the highly conserved amino acid sequence, QVVAG, found in cysteine protease inhibitors including calpain, papain and cathepsin (L, B and H) (SALVESEN *et al.*, 1986). Both HMWK and LMWK bind to platelets, via domain 3 of their heavy chain, and prevent the proteolytically active thrombin from binding to platelets (MELONI & SCHMAIER, 1991).

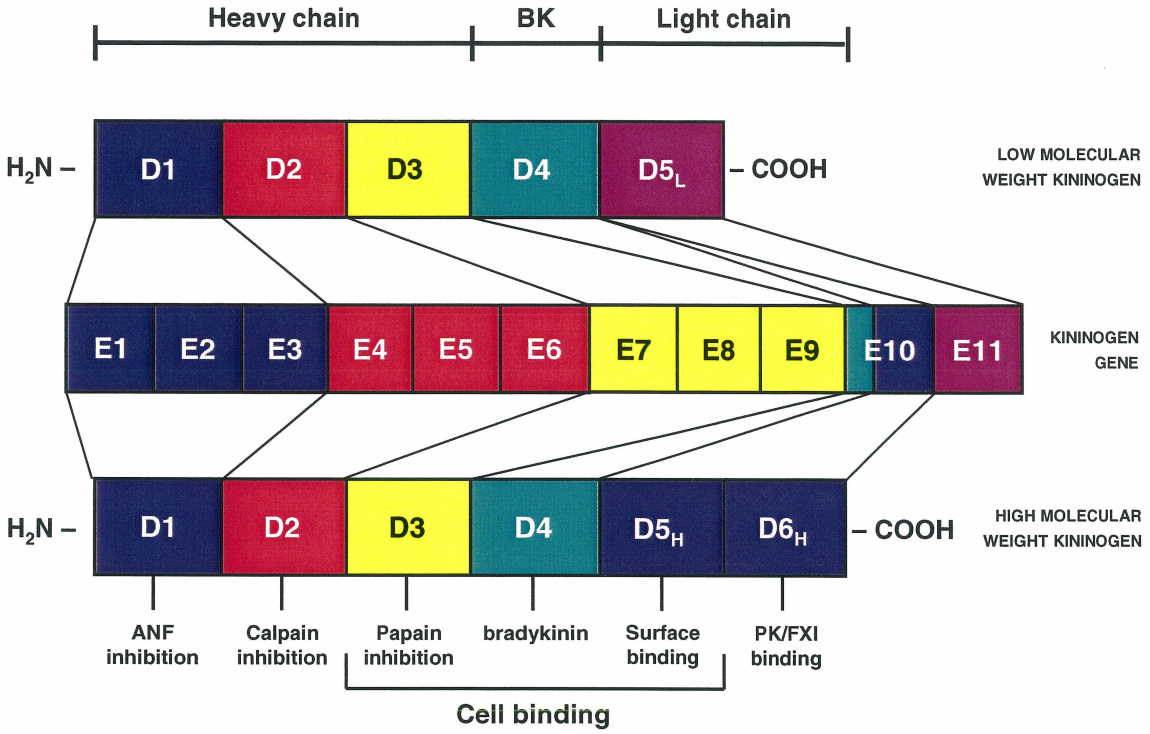

Figure 1. The domain structure of the kininogens. The kininogens are produced by one gene with 11 exons (E1-E11). E1-E3 codes for domain 1 (D1) on both low molecular weight kininogen (LMWK) and high molecular weight kininogen (HMWK). Parts of D1 inhibit atrial naturetic factor. E4-E6 codes for domain 2 (D2), which has papain and unique calpain inhibitory sequences. E7-E9 codes for domain 3 (D3), which has papain inhibitory sequences. Domain 4 (D4) is coded by part of E10; it is the BK sequence on kininogens and the first 12 amino acids of the light chains of LMWK and HMWK. The remainder of E10 codes for HMWK's light chain, which consists of domain 5 ($D5_H$) and domain 6 ($D6_H$). $D5_H$ is an artificial surface binding region; $D6_H$ has the pre-kallikrein and factor XI binding regions. Domains 3 (D3), 4 (D4), and 5 ($D5_H$) on HMWK also participate in cell binding. The E11 codes for the remainder of the unique light chain of LMWK ($D5_L$) *(modified from COLMAN & SCHMAIER, 1997).*

The induction of platelet aggregation by thrombin could be inhibited by the intact HMWK, revealing anti-coagulant properties. In addition, the last 27 amino acids of domain 3, which are contiguous to domain 4, the BK region, are an endothelial cell binding site (COLMAN & SCHMAIER, 1997).

Domain 4, the BK region, has many functions assigned to this nanopeptide in addition to its function as an inhibitor of α-thrombin (HASAN *et al.*, 1996).

The LMWK light chain is 4 kDa and consists of one domain ($D5_L$). Its function is not known. The HMWK light chain is 56 kDa and consists of two domains, 5 ($D5_H$) and 6. The $D5_H$ domain serves as an additional cell binding site on platelets, granulocytes and endothelial cells (COLMAN & SCHMAIER, 1997). The HMWK's domain 6 has a pre-kallikrein and factor XI binding sites. Another characteristic of domain 6 of the HMWK is its ability to selectively interfere with the binding of fibrinogen to integrins on both neutrophils and activated platelets (GUSTAFSON et al., 1989). Thus kininogens can serve as pro-inflammatory proteins by releasing BK, but cleaved kininogens (HMWK) could exhibit antiadhesive and anti-inflammatory properties (COLMAN, 1996).

1.2. Formation of Kinins

Kinins belong to a small family of structurally related 9-11 amino acid peptides including BK, kallidin (KD; lysine-BK; Lys-BK), T-kinin (isoleucine-serine-BK; Ile-Ser-BK) and desArg^{9}kinins (Table 1). Two major biochemical pathways produce kinins (mainly BK and KD), one in the cardiovascular system and a separate system in other tissues (BHOOLA *et al.*, 1992) (Figure 2). In blood, kinin formation is initiated following the activation of

factor XII of the blood clotting sequence (Hageman factor) by contact with surfaces having a negative charge, such as components of tissue matrix (eg. collagen, proteoglycans and heparin) or other negatively charged particles (eg. uric acid, acidic phospholipids, cholesterol sulfate, chondroitin sulfate carrageenan and lipopolysaccharides) (MARGOLIS, 1963; NIEWLAROWSKI *et al.*, 1965). The activated Hageman factor converts the inactive plasma prekallikrein into kallikrein, which normally circulates bound to the HMWK. Kallikrein processing of HMWK liberates BK (PIERCE & GUIMARAES, 1976; BHOOLA *et al.*, 1992; WALKER *et al.*, 1995) (Figure 2). In other tissues (pancreas, salivary glands, colon and skin), proteolytic enzymes activate kallikrein, which act on the LMWK to form mainly KD except in rats where BK rather than KD is produced (BHOOLA *et al.*, 1992; WALKER *et al.*, 1995).

A single gene codes for plasma kallikrein, whereas tissue kallikrein is a member of a multigene family that shows different patterns of tissue-specific gene expression (BHOOLA *et al.*, 1992). T-kinin, another kinin peptide, was identified exclusively in the rat (OKAMOTO & GREENBAUM, 1983) and despite that it could be generated from T-kininogen under the enzymatic action of T-kininogenase *in vitro* (BARLAS *et al.*, 1987), the identity of this enzyme *in vivo* is still unknown (DÉCARIE *et al.*, 1994). T-kininogen consists of two protein isoforms, T-I and T-II kininogens, encoded by two different genes, exhibiting 96% homology. The LMWK and HMWK are derived from a single gene, namely gene K, as a consequence of alternative RNA processing events. The K-and T-kininogen genes first arose by a duplication of an ancestor gene, followed by further duplication to form the two T-kininogen genes (NAKANISHI, 1987) (Figure 3).

	1 2 3 4 5 6 7 8 9
Bradykinin (BK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**-OH
Kallidin (KD)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**-OH
T-kinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**-OH
desArg^{9}BK (DBK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH
desArg^{10}KD	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH
desArg^{11}T-kinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH

The C-terminal Arg is removed under the enzymatic action of carboxypeptidases N and M (kininase I) to generate desArg^{9}BK, desArg^{10}KD and desArg^{11}T-kinin.

Table 1. Primary structure of mammalian kinins. Arg: arginine; Pro: proline; Gly: glycine; Phe: phenyl alanine; Ser: serine; Lys: lysine; Ile: isoleucine *(modified from GABRA et al., 2003)*.

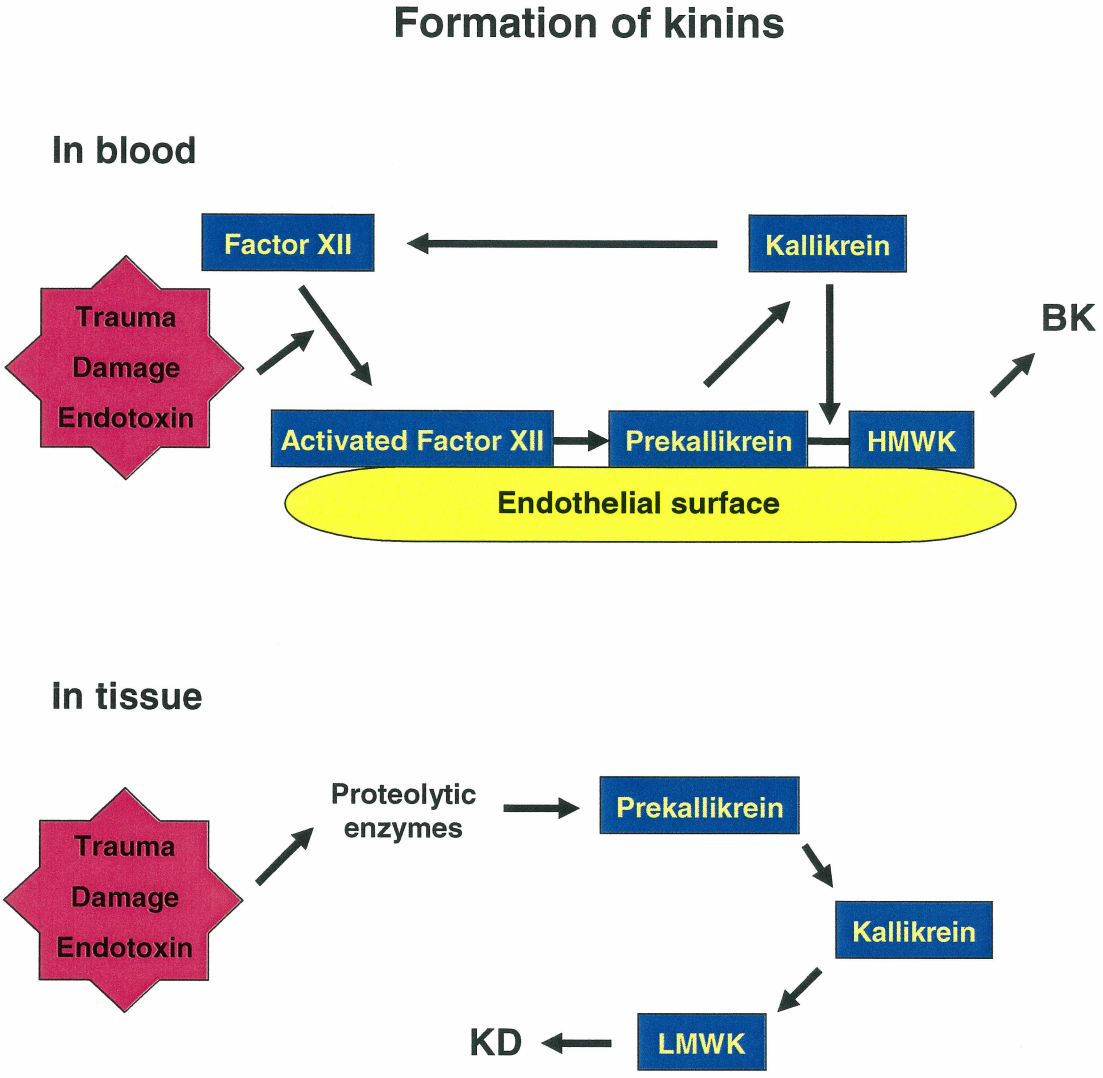

Figure 2. The two main biochemical pathways for formation of kinins in plasma and tissues. In the blood, the initiation step is the activation of Hageman factor XII by damaged surfaces. The subsequent activation of kallikrein liberates bradykinin (BK) from high molecular weight kallikrein (HMWK). In tissues, the precursor for kinin production is the LMWK. In humans, tissue kallikrein liberates kallidin (KD) from low molecular weight kallikrein (LMWK) *(modified from GABRA et al., 2003)*.

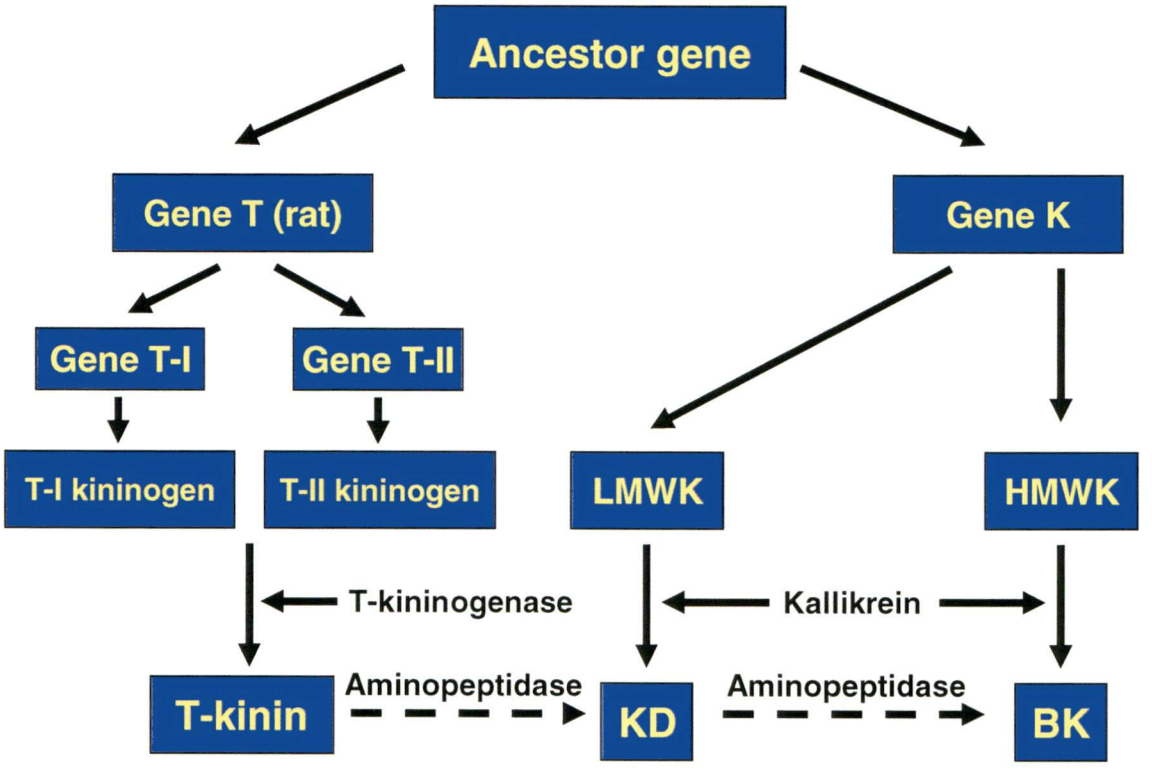

Figure 3. The genes of kinins. BK: bradykinin; KD: kallidin *(modified from GABRA et al., 2003)*.

1.3. Degradation of kinins

Kinins undergo rapid metabolic degradation by a group of amino-, carboxy- and endopeptidases called kininases, found in blood, tissues and biological fluids (BHOOLA *et al.*, 1992) to yield several active and inactive peptides (metabolites) (Figure 4). The half-life of BK is less than 30 sec in plasma or when given intracerebroventricularly (KARIYA *et al.*, 1982). The most physiologically relevant enzymes are kininase I and kininase II. Kininase I peptidase is a family of two carboxypeptidases, kininase I carboxypeptidase N (CPN) of plasma and kininase I carboxypeptidase M (CPM) of cell membrane, that cleave the carboxy terminal Arginine (Arg^9) to generate $desArg^9$BK (DBK) or $desArg^{10}$KD. These enzymes are particularly important as they produce the only metabolites of BK or KD with significant activity.

The Kininase II peptidase also comprises two enzymes, the angiotensin I converting enzyme (ACE) and the neutral endopeptidase (NEP; also called enkephalinase). Both of these enzymes remove the carboxy terminal dipeptide Phe^8-Arg^9 from BK to produce the inactive metabolites BK-(1-7). The kininase II ACE is also known to hydrolyze the Ser^6-Pro^7 bond of BK-(1-7) to give BK-(1-5), which is the final metabolite of BK and DBK. The ACE has been shown as well to slowly convert DBK into BK-(1-5) in human plasma (KUOPPALA et al., 2000) (Figure 5). The KD and T-kinin are also subject to transformation into BK by aminopeptidase activity (KUOPPALA *et al.*, 2000; MURPHEY *et al.*, 2000; CAMPBELL, 2000; COUTURE & LINDSEY, 2000).

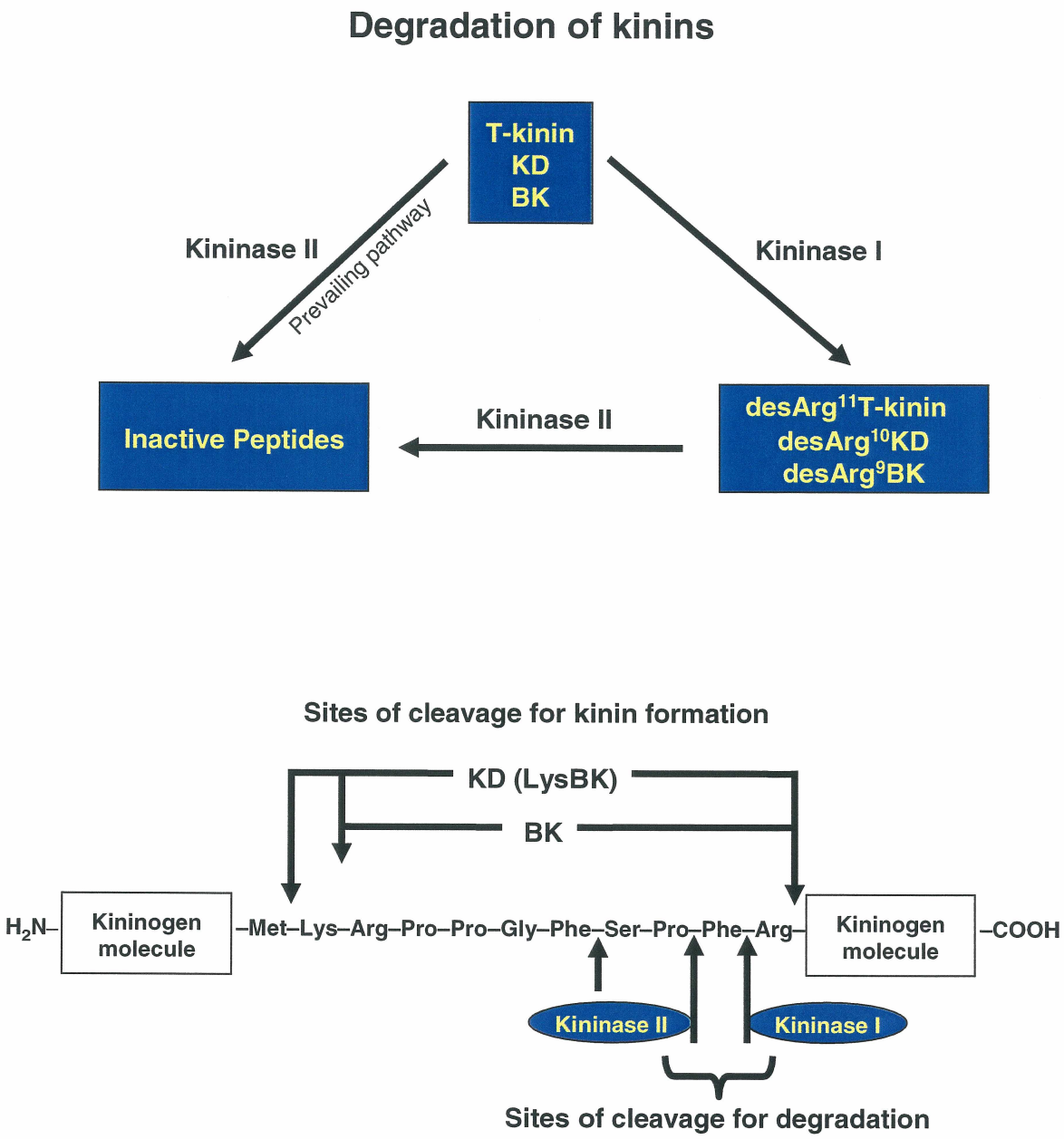

Figure 4. Sites of cleavage for the degradation of kinins. BK: bradykinin; KD: kallidin *(modified from GABRA et al., 2003)*.

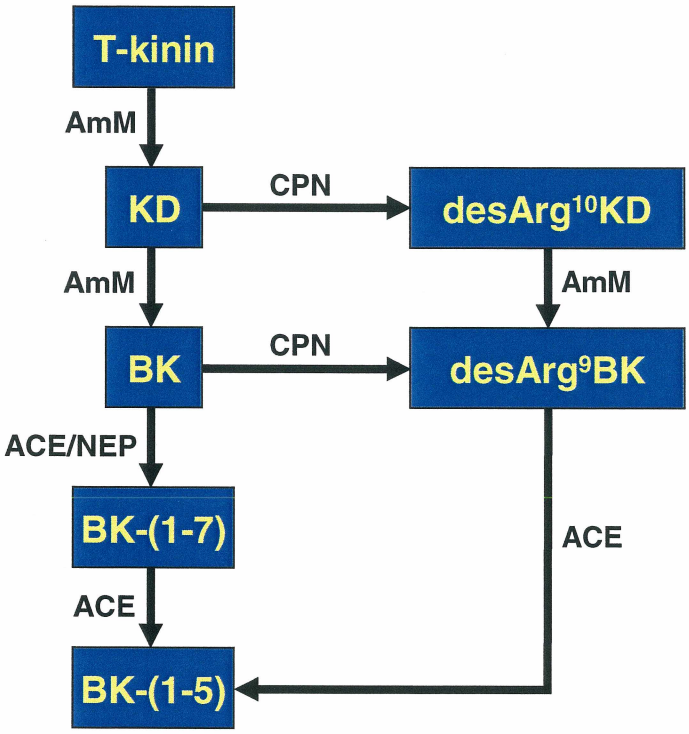

Figure 5. Schematic presentation of the degradation of kinins in human plasma. AmM: aminopeptidase M; CPN: carboxypeptidase N; ACE: angiotensin I converting enzyme; NEP: neutral endopeptidase *(modified from GABRA et al., 2003)*.

1.4. Kinin receptors

Kinins exert their biological effects including vasodilatation, increased vascular permeability, stimulation of pain nerve endings, stimulation of epithelial ion transport and contraction of intestinal and smooth muscles through the activation of two main classes of receptors, denoted B_1 and B_2 receptors (Figures 6, 7 & 8). These receptors have been defined on the basis of their distinct pharmacology using a variety of peptidergic agonists and antagonists. Kinins are the endogenous agonists for the bradykinin B_2 receptor (BKB_2-R) while DBK or desArg^{10}KD are the preferential agonists for the bradykinin B_1 receptor (BKB_1-R) (reviewed by REGOLI & BARABÉ, 1980; Hall, 1992; MARCEAU *et al.*, 1998).).

Both BK receptor genes have been cloned in human and various species. They were found to have seven helix transmembrane domains belonging to the family of G-protein-coupled receptors (GPCR) (MCEACHERN *et al.*, 1991; HESS *et al.*, 1992). The amino acid sequence of human BKB_1-R (353 amino acid protein) is only 36% identical to the amino acid sequence (364 amino acid protein) of the human BKB_2-R (MENKE *et al.*, 1994), while the homology is only 30% between the mouse BKB_2-R (366 amino acid) and BKB_1-R (334 amino acid) (PESQUERO *et al.*, 1996; COUTURE & LINDSEY, 2000) (Table 2).

1.4.1. Genetic structure and genetic variants of the kinin receptors

Using fluorescence in-situ hybridization, MA *et al.* (1994) mapped the BKB_2-R gene to chromosome 14q32. Genomic Southern blot analysis showed that the BKB_2-R is encoded by a single-copy gene and is expressed in most human tissues. With the same approach, CHAI *et al.* (1996) mapped the BKB_1-R gene to chromosome 14q32.1-q32.2, in close proximity to the BKB_2-R gene.

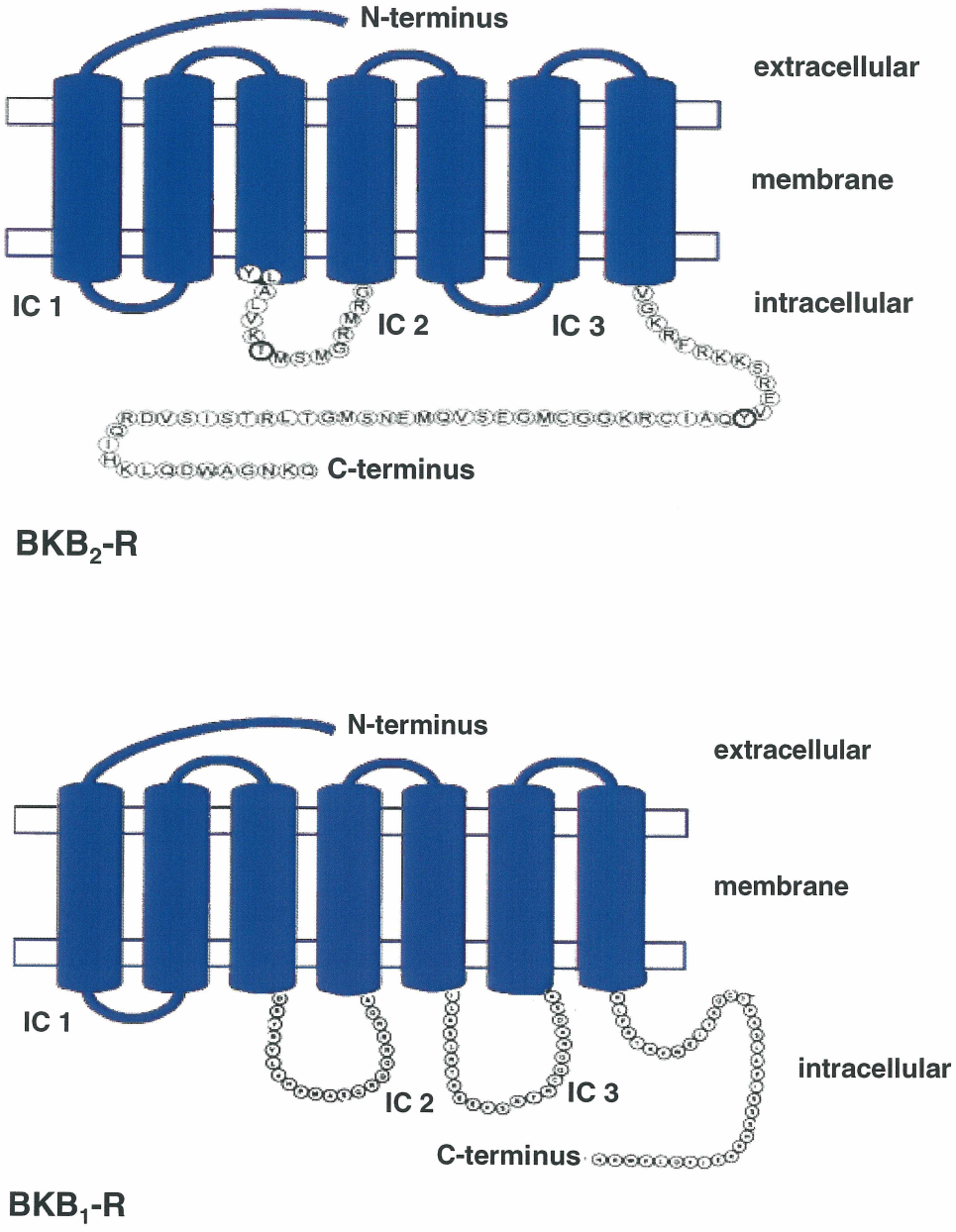

Figure 6. Schematic representation of the intracellular loops 2 and 3 and the C-terminus of the BKB_2-R and BKB_1-R *(modified from PRADO et al., 1998)*.

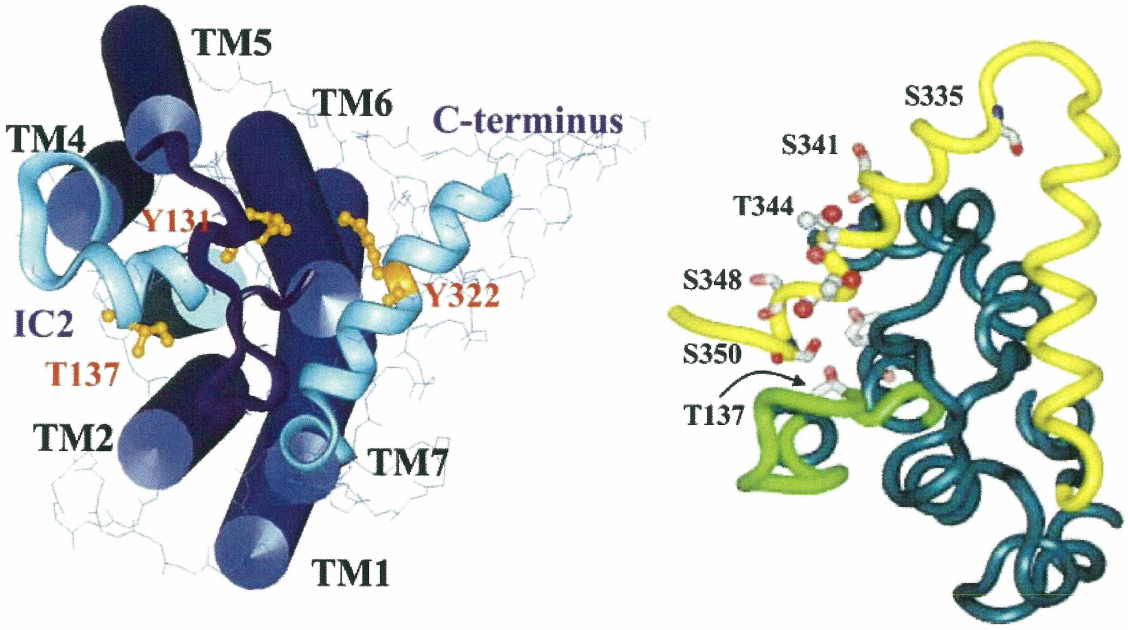

Figure 7. The proximity of intracellular loop 2 (IC2) and C-terminus of BKB_2-R as generated by MD simulations. Left: *bottom view* shows the proximity of Y131 in IC2 to Y322 of the proximal C-terminus. Y131, Y322 and T137 are shown as golden brown residues, the transmembrane helices are shown as blue cylindrical alpha helices, the IC2 and C-terminus are shown as light blue ribbons. **Right:** *bottom view* showing the proximity of T137 of the IC2 to the S/T cluster within the distal C-tail. In particular, S341 and T137 lie in the same plane while S348 projects away. The amino acid are represented by red and gray, IC2 is shown as light green, the C-terminus is shown as yellow and the transmembrane regions as dark green *(taken from PRADO et al., 2002)*.

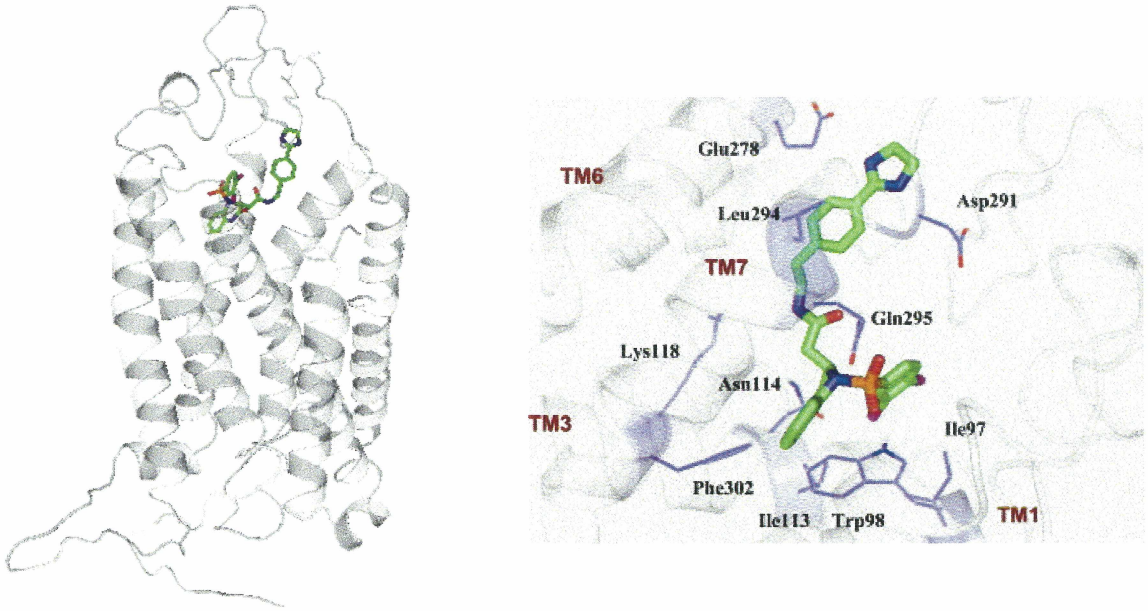

Figure 8. The BKB_1-R generated with the homology module in Molecular Operating Environment (MOE). Left: overall view of a BKB_1-R antagonist into the BKB_1-R model. The antagonist is interacting mainly with transmembrane (TM) helices TM3, TM6, and TM7. **Right:** binding site of the homology model of the BKB_1-R with the antagonist *(taken from HA et al., 2005)*.

	BKB_2-R	BKB_1-R
Family	Rhodopsin superfamily of G protein-coupled receptors (Gαq & Gαi)	Rhodopsin superfamily of G protein-coupled receptors (Gαq & Gαi)
Number of amino acids	366 (rat) 364 (human) 366 (mouse) 367 (rabbit)	337 (rat) 353 (human) 334 (mouse) 352 (rabbit)
Pattern of expression	Constitutive	Inducible
Order of potency of natural agonists	BK >> desArg^{9}BK KD >> desArg^{10}KD	desArg^{9}BK >> BK desArg^{10}KD >> KD
Desensitization	Yes	No
Signal transduction mechanism	PLA_2, PLC, PLD, cAMP, cGMP, ion channnels, PTK, MAP-kinase	PLA_2, PLC, MAP-kinase

Table 2. Properties of kinin receptors. BKB_2-R: bradykinin B_2 receptor; BKB_1-R: bradykinin B_1 receptor; PLA_2: phospholipase A_2; PLC: phospholipase C; MAP-kinase, mitogen-activated protein kinase; PLD: Phospholipase D; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PTK, protein tyrosine kinase *(modified from GABRA et al., 2003)*.

Sequencing results confirmed the locations of these genes and placed them within 12 kb (Figure 9). A three-exon structure for human BKB_2-R gene has been proposed, with the coding region in exon 2 and 3 (KAMMERER *et al.*, 1995). The BKB_1-R gene also contains three exons, separated by two introns. The first and second exons are non-coding, while the third exon contains the full-length coding region (YANG & POLGAR, 1996).

1.4.2. Regulation of kinin receptors expression at transcriptional and post-transcriptional level

1.4.2.1. The BKB_2-R

The nuclear factors involved in regulating the BKB_2-R gene expression are largely unknown. Early studies of the rat BKB_2-R gene revealed lack of a TATA box but identified potential transcription factors such as cAMP response element-binding protein, nuclear transcriptional factor kappa B (NF-κB), AP-1, SP-1 and Egr-1 (PESQUERO *et al.*, 1994). More recently, studies of the promoter region of the rat BKB_2-R gene identified a p53-binding site (a gene that codes for a protein that regulates the cell cycle and hence functions as a tumour suppressor) (SAIFUDEEN *et al.*, 2000). In this study, two p53-like binding sites were identified in the 5′-flanking region of the rat BKB_2-R gene. The AP-1 site, a sequence at 70 bp that is conserved in the murine and human BKB_2-R genes; and the P2 site, a sequence that is not conserved in any of the species (SAIFUDEEN *et al.*, 2000). Although p53 has been implicated to be induced and activated during cellular injury and chronic inflammation (ARAI *et al.*, 1999), it has also been shown to play a role in the regulation of BKB_2-R gene expression in the developing kidney (EL-DAHR *et al.*, 2000). The biological role of the p53-mediated up-regulation of BKB_2-R gene expression still remains unclear.

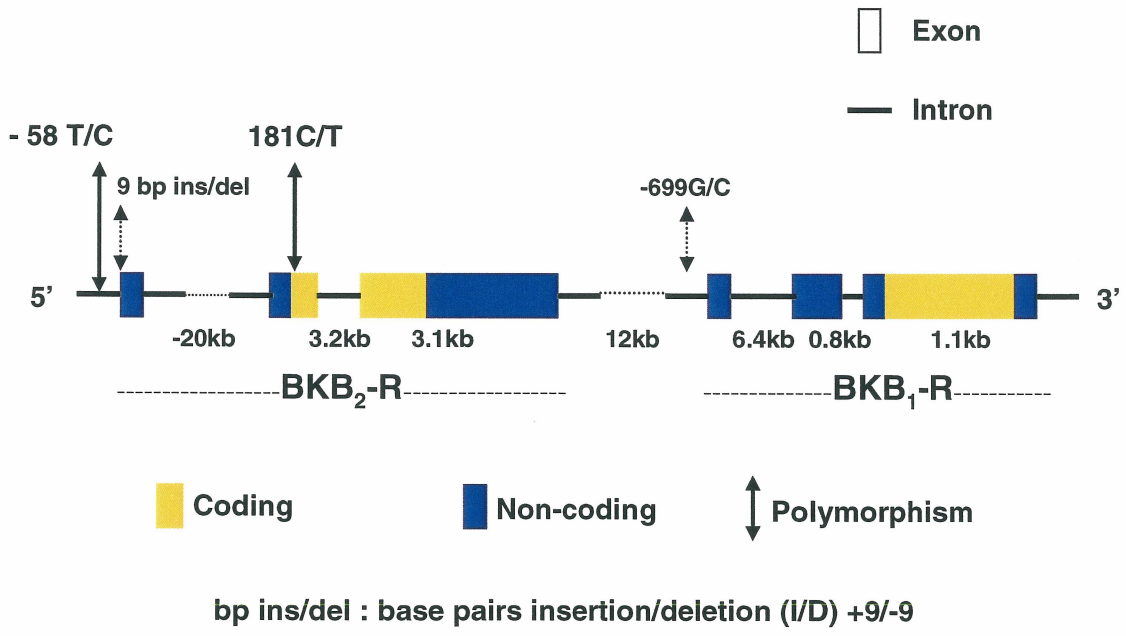

Figure 9. Genomic organization of the human BKB_2-R and BKB_1-R genes at Chromosome 14q32.1. Locations of exons, introns and polymorphisms are illustrated. Shown in the left is the BKB_2-R structure in which exon 1 and a portion of exon 2 (blue boxes) represent the 5′ untranslated region, whereas portions of exon 2 and 3 code for the receptor (yellow boxes). In contrast, the bulk of the translated BKB_1-R gene is located in exon 3 (gray box) with exon 1 and 2 (green boxes) as the 5′ untranslated regions. The length of the introns between the two receptors is indicated *(modified from PRADO et al., 2002)*.

1.4.2.2. The BKB_1-R

The BKB_1-R gene expression is highly regulated and represents a good model for studies of inducible genes. It is present as a single copy which spans more than 10 kb and includes three exons interrupted by two introns (Figure 9). While the 5′ untranslated region is distributed on all three exons, the coding region is located entirely on the third exon. Two distinct functional promoters are found in the human BKB_1-R gene. Exon and intron-exon boundaries, and 5′, 3′ flanking regions were sequenced. While the 5′ untranslated region proved to be distributed on all three exons, the coding region was located entirely in the third exon. The exon-intron junction sequences are highly conserved. Primer extension analysis mapped the transcriptional initiation site 21 base pairs (bp) down-stream of a TATA sequence (a deoxyribonucleic acid (DNA) sequence found in the promoter region) of most genes and downstream of numerous transcription factor binding motifs (Figure 10) (BACHVAROV *et al.*, 1996; YANG & POLGAR, 1996). From studies by YANG & POLGAR (1996), a positive regulatory element (PRE) was located at position -604 to -448 bp up-stream of the transcription start site (Figure 10).

This PRE contains a classic powerful enhancer and a negative regulatory element, at position -682 to -604. The negative element ablates the function of the enhancer. The region of the enhancer and silencer was minimized to a 100-bp element and a 78-bp fragment, respectively. Transient transfection of the enhancer construct into a variety of cell types showed that this enhancer is cell-type specific (YANG & POLGAR, 1996). In the characterization of the enhancer two motifs were found to be essential for full enhancer activity. Gel shift and antibody supershift assays determined that a transcriptional factor AP-1 binds one of these motifs. The nuclear protein, which binds the other motif, has yet to be identified. Both factors are the critical regulators for this enhancer activation.

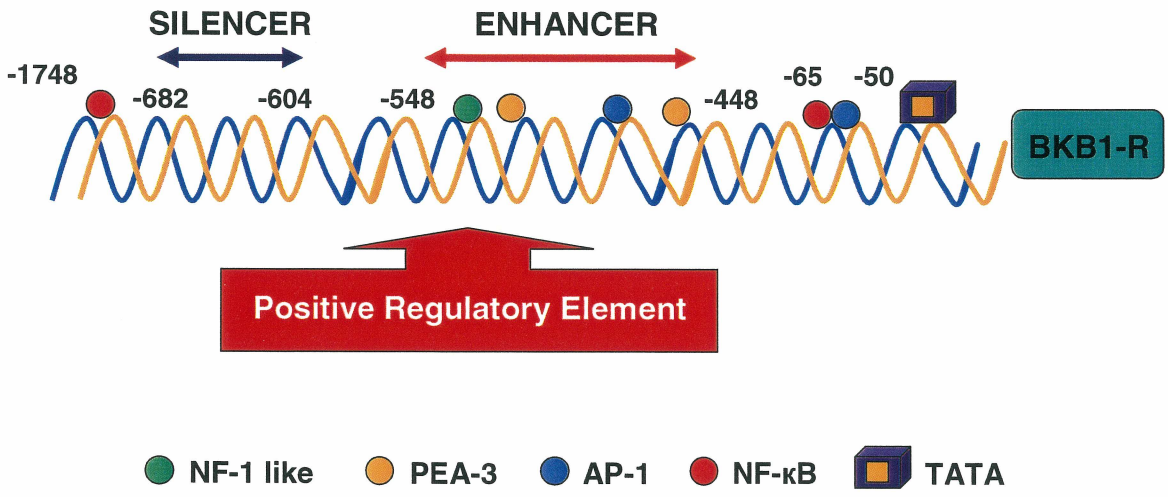

Figure 10. Representation of the human BKB_1-R gene promoter with its putative nuclear factor binding sites. The variously colored circles represent the reported locations of the given *cis* elements which includes the transcription factors NF-1-like, PEA-3, AP-1 and nuclear transcriptional factor kappa B (NF-κB). The consensus TATA box (a DNA sequence *(cis-element)* found in the promoter region of most genes and considered to be a promoter sequence) is indicated by a box. The enhancer and the silencer regions are indicated by arrows (↔). A positive regulatory element (PRE) is located at position -604 to -448 upstream of the transcription start site *(modified from PRADO et al., 2002)*.

1.4.3. Kinin receptors signaling

Immunoprecipitation of photoaffinity-labeled G-proteins demonstrated BKB_1-R coupling to Gαq/11 and Gαi(1,2) (AUSTIN *et al.*, 1997). A similar approach also showed Gαq and Gαi subunit coupling to the BKB_2-R (DE WEERD & LEEB-LUNDBERG, 1997). With regard to Gαi, perhaps the most direct evidence of coupling to BKB_2-R came from knockdown studies in Rat-1 cells (YANG *et al.*, 1999). Knockdown of either Gαi(2) or Gαi(3) protein production did not affect the binding of BK. In the Gαi(2)-depleted cells, BK-induced arachidonate (ARA) release was reduced by more than 60%. In the Gαi(3)-depleted cells, BK-induced ARA release was decreased by over 50%. Direct evidence also exists that the Gαq protein family interacts with BKB_2-R and performs an important role in BK-induced total inositol 1,4,5-triphosphate (IP3) formation. This was shown with Gαq-null embryonic stem cells (RICUPERO *et al.*, 1997). Their results demonstrated that more than one member of the Gαq family is involved in phosphatidyl inositol (PI) turnover. The Gβγ dimer, dissociated from activated Gαi-protein heterotrimer, was also reported as the signal transducer in Gαi-sensitive inositol phosphate formation (CAMPS *et al.*, 1992). Clearly, the BKB_2-R interacts with at least two families of Gα subunits and perhaps also βγ subunits.

As discussed above, BK is the primary endogenous agonist of the BKB_2-R while DBK and desArg^{10}KD are the favoured agonists for the BKB_1-R. Upon activation, the BKB_2-R initiates an array of intracellular and intercellular responses which vary with cell and tissue type. Depending upon the cell type, BK induces excitability, contraction, cell division, permeability and release of a variety of biologically active agents (GOLDSTEIN & WALL, 1984; REGOLI, 1984; VINCENTINI & VILLEREAL, 1984; GAGINELLA & KACHUR, 1989; ROBERTS, 1989). Early post-binding events include an increase in cytosolic calcium (Ca^{2+}), activation of G-protein, guanylate cyclase (GC), and phospholipases C (PLC),

D (PLD), and A_2 (PLA_2) (BURCH & AXELROD, 1987; KREMER *et al.*, 1988; VOYNO-YASENETSKAYA *et al.*, 1989; YANAGA *et al.*, 1991; TAYLOR *et al.*, 1992; RICUPERO *et al.*, 1993; 1997; LEE *et al.*, 2000; ZHOU *et al.*, 2000; EXTON, 2002). The signaling actions of BKB_1-R in response to DBK or desArg^{10}KD are very similar to those of BKB_2-R with respect to the activation of PI turnover, ARA release, Ca^{2+} mobilization and the induction of the immediate early gene, c-fos. (CAHILL *et al.*, 1988; MENKE *et al.*, 1994; JONG *et al.*, 1996; ZHOU *et al.*, 1999; SCHAEFFER *et al.*, 2001). Like BK, DBK also induces protein formation and cell division (GOLDSTEIN & WALL, 1984; BENY *et al.*, 1987; CHURCHILL & WARD, 1987; VIANNA & CALIXTO, 1998; PRAT *et al.*, 1999). The DBK and desArg^{10}KD activate the BKB_1-R with different specificities depending on the species (HESS *et al.*, 1996; PESQUERO *et al.*, 1996).

The signals generated by the BKB_1-R and the BKB_2-R at first glance appear to be identical. Both receptors induce an increase of cytosolic Ca^{2+}, activate PLC and couple to the G-proteins Gαq, Gαi2 and Gαi3 (YANAGA *et al.*, 1991; LIAO & HOMCY, 1993; AUSTIN *et al.*, 1997; XIE *et al.*, 2000). However, a closer inspection reveals that the kinetics of the increase of $[Ca^{2+}]_i$ is quite distinct, suggesting the intracellular signaling that induces the increase of cytosolic Ca^{2+} are also distinct (TROPEA *et al.*, 1993; MARSH & HILL, 1994; MATHIS *et al.*, 1996). With regard to $[Ca^{2+}]_i$, the BKB_1-R utilizes largely extracellular Ca^{2+} (ZHOU *et al.*, 2000) while BKB_2-R utilizes mostly Ca^{2+} located within intracellular compartments, likely through IP3-gated Ca^{2+} channels in the endoplasmic reticulum (MOMBOULI & VANHOUTTE, 1995) (Figure 11).

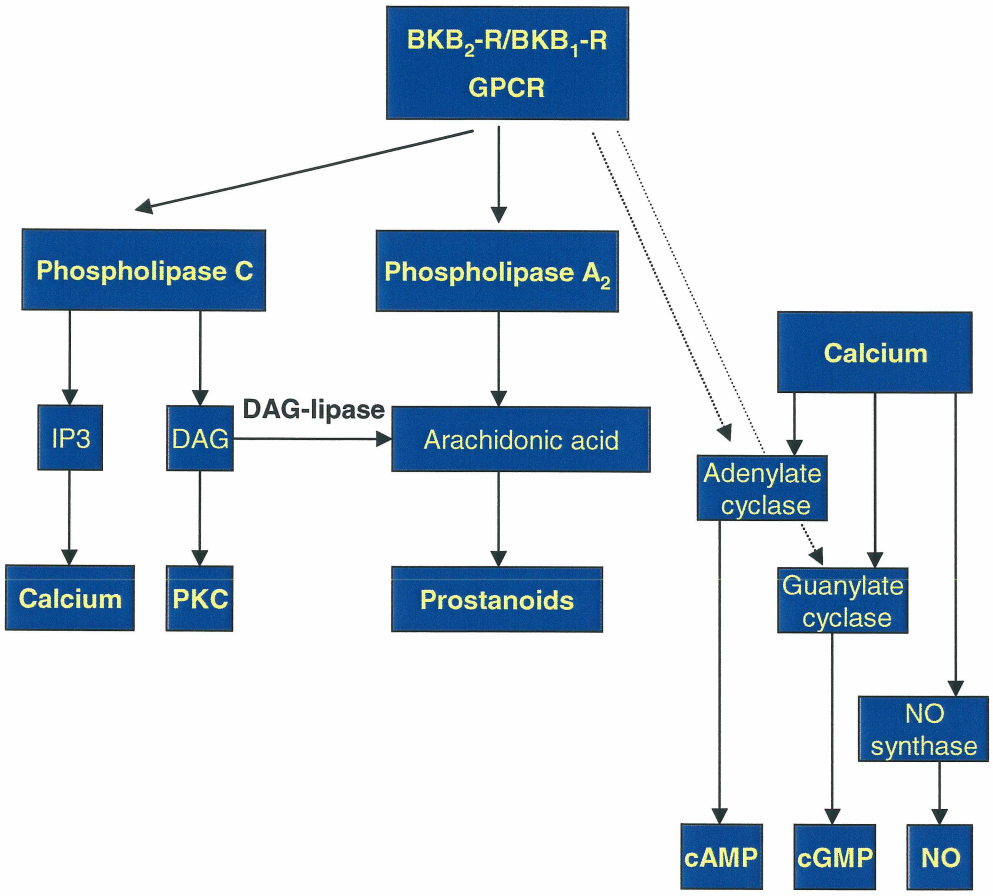

Figure 11. Signal transduction pathways for the kinin receptors. GPCR: G-protein-coupled receptor; PKC: protein kinase C; IP3: inositol 1,4,5-triphosphate; DAG: diacylglycerol; NO: nitric oxide *(modified from GABRA et al., 2003)*.

These data suggest that different signaling paths are utilized by these two receptors to achieve increases in $[Ca^{2+}]_i$ which in turn, stimulates the formation of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and nitric oxide (NO). Activation of the BKB_2-R leads to processes that can be considered as anti-fibrotic. For example, the activation of the BKB_2-R does not stimulate collagen or connective tissue growth factor (CTGF) synthesis in fibroblasts while the BKB_1-R does (RICUPERO *et al.*, 2000; YU *et al.*, 2002). Also, treatment with ACE inhibitors, blocking the production of angiotensin II and promoting the accumulation of BK, has clearly been shown to be anti-fibrotic (BORDER & NOBLE, 2001). This is in contrast to BKB_1-R, which upon activation by $desArg^{10}KD$ stimulates type I collagen synthesis and raises α1(I) collagen mRNA and CTGF mRNA (RICUPERO *et al.*, 2000). The $desArg^{10}KD$, tumour growth factor-β (TGF-β) and angiotensin II have also been reported to increase CTGF production in fibroblasts (RICUPERO *et al.*, 2000; YU *et al.*, 2002). Also, IMR90 cells, human embryonic lung fibroblasts, exhibit mitotic and collagen synthetic responses to DBK (GOLDSTEIN & WALL, 1984). Thus, BKB_1-R plays a distinct, stimulatory role in wound repair and is pro-fibrotic.

1.4.4. Physiological functions of the Kinin receptors

1.4.4.1. The BKB_2-R

The BKB_2-R is constitutively expressed and accounts for the majority of the acute pharmacological actions of kinins (WALKER *et al.*, 1995). This receptor subtype has a widespread distribution and is notably on endothelial cells, smooth and striated muscle cells, fibroblasts, mesangial and epithelial cells and certain neurons and hematopoetic cells (eg. neutrophils) (BHOOLA *et al.*, 1992; HALL, 1992). Many pharmacological studies using

agonists and antagonists militate in favour of the existence of heterogenous populations of BKB_2-R; these homologues or isoforms of the BKB_2-R seem rather related to animal species. For example, the rabbit BKB_2-R subtype is pharmacologically similar to that of human but different from that of the rat or the guinea-pig (REGOLI *et al.*, 1994).

Kinins are the endogenous agonists for the BKB_2-R. The preferred agonist for the BKB_2-R is BK, but KD also acts via BKB_2-R both by direct interaction and potentially via conversion to BK by aminopeptidases (HALL, 1992). Evidence suggests that kallikreins and some other proteases activate human BKB_2-R directly, independent of BK release. Thus, the BKB_2-R might belong to a new group of serine protease-activated receptors (HECQUET et al., 2000). Highly potent and selective peptide and non-peptide agonists and antagonists are available for the BKB_2-R (REGOLI *et al.*, 1998; ALTAMURA *et al.*, 1999) (Table 3).

The BKB_2-R is involved in the acute phase of inflammatory and pain response (ELLIOTT *et al.*, 1960; Dray & Perkins, 1993; Dray, 1997). This is likely to occur because BKB_2-R function is controlled by short-term mechanisms involving fast ligand dissociation, receptor desensitization and internalization, and on long-term stimulation, down-regulation (MUNOZ *et al.*, 1993; FAUSSNER *et al.*, 1999; MARCEAU *et al.*, 2001).

1.4.4.1.1. The BKB_2-R and vasculature

In the treatment of hypertension and other cardiovascular diseases, including cardiac insufficiency, myocardial infarction and diabetic nephropathy, the ACE inhibitors (captopril, enalapril and others) are clinically used with good results. These inhibitors, not only prevent the transformation of angiotensin I into angiotensin II (a potent vasoconstrictor), but also reduce the metabolism of endogenous kinins (LINZ *et al.*, 1995).

	Peptides	Non-peptides
Agonists	**BK** **Lys-BK (KD)**	**FR 190997**
Antagonists	**[D-Phe^7] BK** **D-Arg [Hyp^3, Thi^{5,8}, D-Phe^7] BK** **Icatibant (Hoe 140)** **Men 11270** **NPC 17731** **NPC 17761**	**WIN 64338** **FR 173657** **LF 16.0335** **LF 16.0687** **Bradyzide**

FR 190997	8-[2,6-dichloro-3-[*N*-[(E)-4-(*N*-methylcarbamoyl) cinnamidoacetyl]-*N* methylamino] benzyloxy]-2-methyl-4-(2-pyridylmethoxy) quinoline (*selective non-peptidic BKB_2-R agonist*) (ARAMORI *et al.* 1997a).
Hoe 140	D-Arg [Hyp^3, Thi^5, D-Phe^7, Oic^8] BK (*selective peptidic BKB_2-R antagonist*) (HOCK *et al.*, 1991; WIRTH *et al.*, 1991)
Men 11270	The cyclic constrained derivative of Hoe 140 (D-Arg-Arg-Pro-Hyp-Gly-Thi-c(Dab-D-Tic-Oic-Arg)c(7 γ-10 α)) (MEINI *et al.*, 1999).
NPC 17731	D-Arg^0 [Hyp^3, D-Hyp-E(*trans*-propyl)^7, Oic^8] BK (*selective peptidic BKB_2-R antagonist*) (KYLE *et al.*, 1991).
NPC 17761	D-Arg^0 [Hyp^3, D-Hyp-E(*trans*-thio-phenyl)^7, Oic^8] BK (*potent, selective and competitive peptidic BKB_2-R antagonist*) (KYLE *et al.*, 1991).
WIN 64338	([[4-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl]amino]phenyl]methyl]tributylphosphoniumchloride monohydrochloride) (*selective competitive non-peptidic BKB_2-R antagonist*) (SALVINO *et al.*, 1993; SAWUTZ *et al.*, 1994).
FR 173657	((*E*)-3-(6-acetamido-3-pyridyl)-N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-N-[methylaminocarboxylmethyl]-acrylamide) (*potent, orally active and selective non-peptidic BKB_2-R antagonist*) (ARAMORI *et al.* 1997b).
LF 16.0335	(1-[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichloro-phenyl]-sulphonyl]-2(S)-[[4-[4-(aminoiminomethyl)phenyl-carbonyl]piperazin-1-yl]carbonyl]pyrrolidine) (*potent, selective and competitive non-peptidic BKB_2-R antagonist*) (PRUNEAU *et al.*, 1998).
LF 16.0687	(1-[[2,4-dichloro-3-[[(2,4-dimethylquinolin-8-yl)oxy] methyl] phenyl]-sulphonyl]-*N*-[-3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]-propyl]-2(*S*)-pyrrolidinecarboxamide) (*potent, selective and competitive non-peptidic BKB_2-R antagonist*) (PRUNEAU *et al.*, 1999).
Bradyzide	((2S)-1-[4-(benzhydrylthiosemicarbazido)-3-nitrobenzenesulfonyl]-pyrrolidine-2-carboxilic acid {2-(2-dimethylaminomethyl)-methylamino]ethyl}amide) (*potent non-peptidic BKB_2-R antagonist with long-lasting oral activity*) (BURGESS *et al.*, 2000).

Table 3. Agonists and antagonists for the BKB_2-R. *(modified from GABRA et al., 2003).*

Other studies have demonstrated that ACE inhibitors block the BKB_2-R desensitization, thereby potentiating bradykinin beyond blocking its hydrolysis. In addition, angiotensin (1-7) (an ACE inhibitor) was shown to stimulate BK release via angiotensin II type 2 receptors (TOM *et al.*, 2001). Furthermore, some recent studies showed that, in patients with stroke and long-standing hypertension, the auto-regulation of cerebral blood flow induced by the ACE inhibitor captopril is mediated via the action of BK on BKB_2-R (TAKADA *et al.*, 2001). Moreover, DUKA *et al.*, (2001) found that BKB_2-R-knockout mice had a higher baseline blood pressure compared to wild-type controls. On the other hand, RHALEB *et al.*, (2001) proved that endogenous kinins do not participate in the maintenance of normal blood pressure or antagonize the development of hypertension induced by chronic infusion of angiotensin II, a high salt diet or deoxycorticosterone acetate (DOCA) plus high salt diet. However, kinins appear to play an important role in the antihypertensive effect of ACE inhitors in DOCA-salt hypertension (RHALEB *et al.*, 2001).

1.4.4.1.2. The BKB_2-R and neurogenic inflammation

The BKB_2-R is involved in most signs of acute inflammatory vasculature, including increased vascular permeability, venoconstriction, arterial dilatation and oedema (HALL, 1992). The production of oedema and vascular permeability was found to be mainly mediated through the constitutive BKB_2-R in several models of acute visceral and cutaneous inflammation such as pancreatitis and cystitis or whether it occurs in response to treatment with carrageenan or collagenase (GRIESBACHER & LEGAT, 1997, 2000). Activation of the BKB_2-R results in vasodilation due to stimulation of nitric oxide synthase (NOS) and PLA_2 in vascular endothelial cells of pre-capillary arteries and arterioles to produce

NO and prostaglandin I_2 (PGI_2) (REGOLI, 1984; BHOOLA *et al.*, 1992) (Figure 12). It has been suggested that the metabolism of BK into DBK and L-arginine provides the important precursor L-arginine for further NO production (HALL, 1992). In addition, on the post-capillary veins and veinules, BK induces a Ca^{2+}-dependent activation of contractile proteins and the consequent contraction of vascular endothelial cells (Figure 13). This results in the formation of fenestrations in the wall of microvessels, principally veinules, which enables extravasation of blood constituents including specific cellular elements (REGOLI, 1984). Taken together, the vasodilatation of pre-capillary arterioles and the constriction of post-capillary veinules result in the increase of hydrostatic pressure in the capillaries leading to plasma extravasation (D'ORLÉANS-JUSTE *et al.*, 1996).

The BK is also known to facilitate the release of pro-inflammatory neuropeptides including substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) from rat sensory neurones in culture (VASKO *et al.*, 1994). The SP and NKA contribute to inflammation by contracting endothelial cells in veinules thus inducing plasma extravasation. SP and NKA also induce vasodilation via the release of NO from endothelial cells. CGRP contributes to neurogenic inflammation by producing dilatation of arterioles and increasing blood flow (WALKER *et al.*, 1995). The increase of BK-induced release of sensory neuropeptides is generally augmented by prostaglandins and reduced by cyclooxygenase inhibitors (ANDREEVA & RANG, 1993). Post-ganglionic sympathetic nerve fibres may also be excited by BK during inflammation leading to plasma extravasation by prostaglandins release (vasodilatation) and noradrenaline release (vasoconstriction) (GREEN *et al.*, 1993).

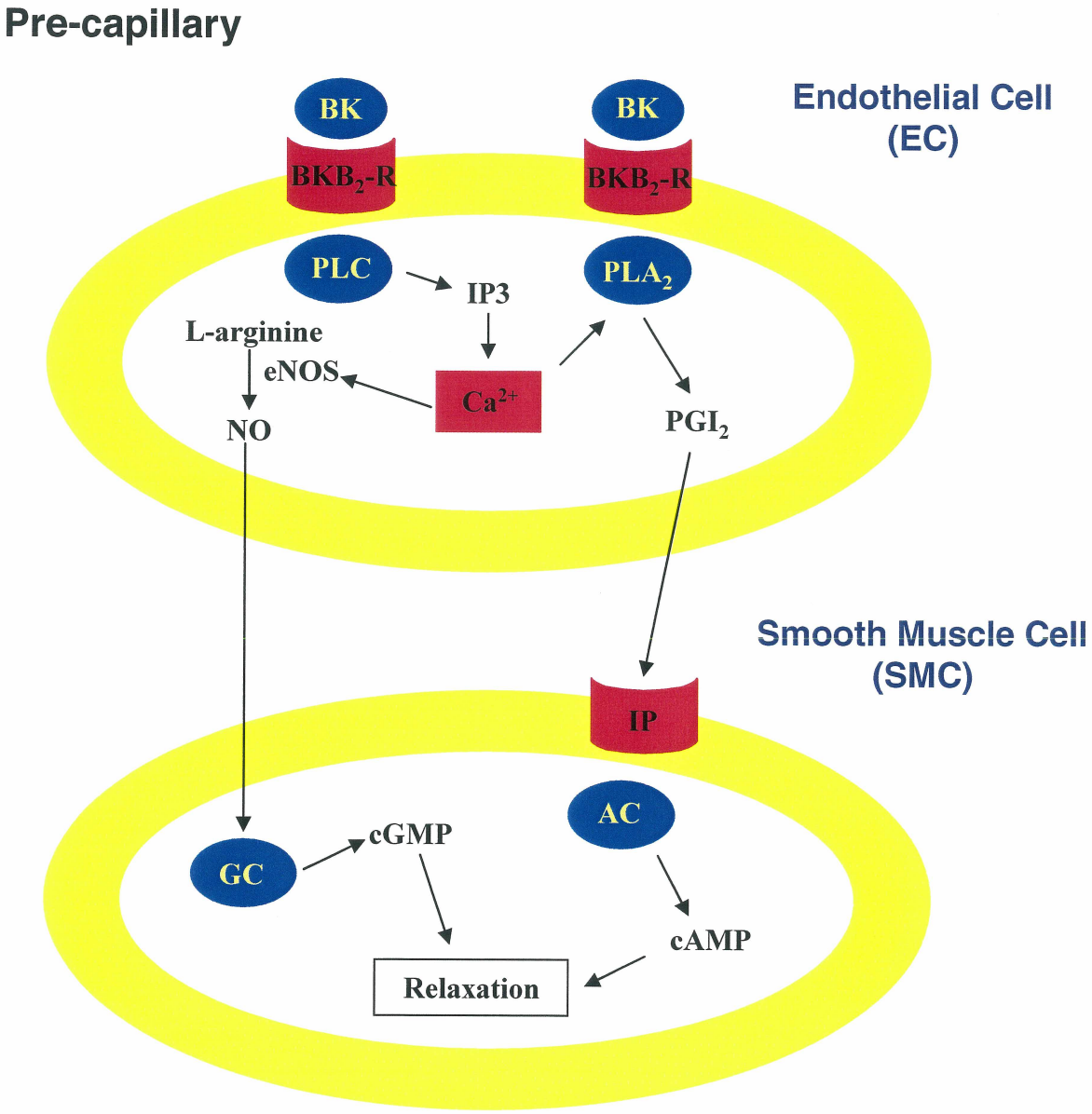

Figure 12. Mechanisms of action of BK on pre-capillary vessels. PLC: phospholipase C; PLA_2: phospholipase A_2; IP3: inositol 1,4,5-triphosphate; NO: nitric oxide; eNOS: endothelial nitroc oxide synthase; Ca^{2+}: Calcium; PGI_2: prostaglandin I_2; IP: prostanoid PGI_2 receptor; GC: guanylate cyclase; AC: adenylate cyclase; cGMP: cyclic guanosine monophosphate; cAMP: cyclic adenosine monophosphate *(modified from GABRA et al., 2003)*.

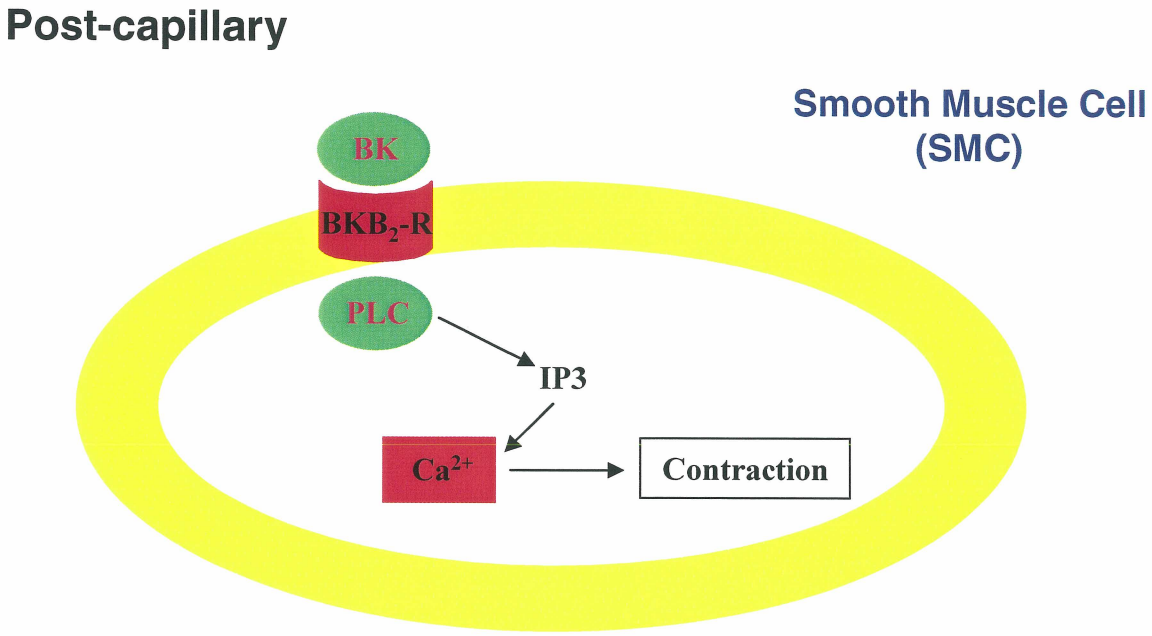

Figure 13. Mechanisms of action of BK on post-capillary vessels. PLC: phospholipase C; IP3: inositol 1,4,5-triphosphate; Ca^{2+}: Calcium *(modified from GABRA et al., 2003)*.

1.4.4.1.3. The BKB_2-R and peripheral inflammatory pain

Exogenous kinins were found to produce pain perception stimulating the BKB_2-R in the blister base of human skin (WHALLEY *et al.*, 1987) and in rat tissues (STERANKA *et al.*, 1988). The activation of BKB_2-R, constitutively expressed in primary sensory neurones (STERANKA *et al.*, 1988; BURGESS *et al.*, 1989; DRAY *et al.*, 1992), promotes polymodal nociceptor activation and hyperalgesia through the production of diacyl glycerol (DAG) and activation of protein kinase C (PKC) (DRAY *et al.*, 1992). Moreover, BK can sensitize nociceptors following the release of prostaglandins, cytokines and NO either from sensory neurones, endothelial and immune cells or fibroblasts in addition to interaction with mast cells mediators as histamine and serotonin (DRAY & PERKINS, 1997). Stimulation of sympathetic nerves, to release prostanoids or other mediators that sensitize nociceptors, also contributes to BK-induced hyperalgesia (TAIWO & LEVINE, 1988). These pieces of evidences could explain the antinociceptive effect of BKB_2-R antagonists in acute inflammatory hyperalgesia models (HEAPY *et al.*, 1991; PERKINS *et al.*, 1993; RUPNIAK *et al.*, 1997; CALIXTO *et al.*, 2000) and the alterations of the nociceptive responses in BKB_2-R knockout mice (BOYCE *et al.*, 1996; RUPNIAK *et al.*, 1997).

1.4.4.1.4. The BKB_2-R and immune cells

Activation of the BKB_2-R has been reported to inhibit polymorphoneuclear chemotaxis (ROCH-ARVEILLER *et al.*, 1981) and this receptor is considered to have a limited role in the cellular component of the inflammatory response involving leukocyte recruitment within the microcirculation (McLEAN *et al.*, 2000). At very high doses, BK can cause

BKB_2-R-mediated leukocyte recruitment through the release of platelet-activating factor possibly from the endothelium and macrophages (SHIGEMATSU *et al.*, 1999).

1.4.4.1.5. The BKB_2-R and modulation of central pain

The CNS contains all of the components of the kallikrein-kinin system. The BKB_2-R has been identified in mouse and rat brain tissue cultures (LEWIS *et al.*, 1985) and in various regions of mammalian brain and spinal cord. The highest density of BK binding sites was found in the pons, medulla oblongata and spinal cord (FUJIWARA *et al.*, 1989). A moderate density was found in the cerebral cortex and hippocampus, with a lower density being found in other brain regions (FUJIWARA *et al.*, 1989). The majority of binding sites identified in these autoradiography studies, were localized to cerebral blood vessels, however in the spinal cord, the BKB_2-R has been identified in the superficial layers of the dorsal horn confined to the terminals of the primary sensory Aδ and C-fibres and spinal noradrenergic terminals (STERANKA *et al.*, 1988; FUJIWARA *et al.*, 1989). When administered into the cerebral ventricles, BK caused an antinociceptive effect through a noradrenergic mechanism in rabbits (RIBEIRO & SILVA, 1973) or NO in mice (GERMANY *et al.*, 1996) and by stimulation of the BKB_2-R in rat brain (PÉLA *et al.*, 1996). Nociceptive behavioural activity was increased following intra-thecal administration of BK in the awake rat (LANEUVILLE & COUTURE, 1987). This nociceptive behavioural excitation lasted less than 1 min and was followed by a longer period of quietness (10-15 min), which was associated with an increase in the tail flick latency in the rat (LANEUVILLE & COUTURE, 1987; LANEUVILLE *et al.*, 1989). The initial nociceptive response was described as a direct action of BK on the BKB_2-R located on the sensory terminals projecting to the superficial laminae of the spinal cord. On the other

hand, the antinociceptive response (also mediated by the BKB_2-R) was shown to be due to release of noradrenaline from descending inhibitory neurons projecting to the dorsal horn, with the subsequent activation of α-adrenoreceptors (LANEUVILLE *et al.*, 1989). The BK-induced stimulation of the central BKB_2-R may be important in mediating the pain of migraine, as well as pain in other cerebrovascular disorders and the neurogenic inflammation that follows various forms of brain injury (MACFARLANE *et al.*, 1991).

1.4.4.2. The BKB_1-R

The BKB_1-R subtype is generally absent in normal tissues and healthy animals but can be expressed or up-regulated both *in vitro* and *in vivo* during tissue injury, following treatment with bacterial endotoxins, lipopolysaccharide (LPS) and cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (MARCEAU *et al.*, 1980; REGOLI *et al.*, 1981; deBLOIS *et al.*, 1988, MARCEAU *et al.*, 1998) as well as in animals diagnosed with an established infection (SIEBECK *et al.*, 1998). Some exceptions however exist. In dogs, the BKB_1-R seems to be constitutive since injection of its selective agonist, DBK, causes hypotension, natriuria and renal vasodilation (NAKHOSTINE *et al.*, 1993). Furthermore, complex hemodynamic response was obtained in cats following the injection of DBK in the pulmonary circulation (DeWitt *et al.*, 1994). In addition, WOTHERSPOON & WINTER (2000) demonstrated that the BKB_1-R is constitutively expressed in the naive rat sensory nervous system (laminae 1 and 2 of the dorsal horn of the spinal cord and on the peripheral nerve terminals in the bladder).

Several peptidic agonists and antagonists have been developed for BKB_1-R (Table 4). However, the only potent and selective non-peptidic BKB_1-R antagonists are benzodiazepine derivatives from Merck Research Laboratories (SU *et al.*, 2003).

Agonists	Antagonists
desArg^{9}BK (DBK)	[Leu^{8}] desArg^{9}BK
desArg^{10}KD	Lys-[Leu^{8}] desArg^{9}BK *
Sar [D-Phe^{8}] desArg^{9}BK	desArg^{10}Hoe-140 **
	B-9958 ***
	R-715 ***
	R-954***

B-9958	Lys-Lys-[Hyp^{3}, Cpg^{5}, D-Tic^{7}, Cpg^{8}] desArg^{9}BK
R-715	Ac-Lys-[D-ß Nal^{7}, Ile^{8}] desArg^{9}BK
R-954	Ac-Orn-[Oic^{2}, α-Me Phe^{5}, D-ß Nal^{7}, Ile^{8}] desArg^{9}BK

Table 4. Peptidic agonists and antagonists for the BKB_1-R *(modified from GABRA et al., 2003).*

* **Regoli & Barabé, 1980**

** **Wirth *et al.*, 1991**

*** **Regoli *et al.*, 1998; 2001; Neugebauer *et al.*, 2002**

Pharmacological evidence suggests the existence of interspecies BKB_1-R subtype. The BKB_1-R found in dog, rat, mouse and hamster is a subtype that differs from the human, rabbit and pig BKB_1-R (REGOLI *et al.*, 2001; HESS, *et al.*, 2001).

Contrary to the BKB_2-R, the BKB_1-R participates in the chronic phase of the inflammatory and pain response (DRAY & PERKINS, 1993; DRAY, 1997). The BKB_1-R elicits persistent responses and signalling that are subject to very limited desensitization, and receptor internalization with very low ligand dissociation (MARCEAU *et al.*, 1998). In addition, upon long-term exposure to its endogenous agonist, the BKB_1-R is up-regulated (FAUSSNER *et al.*, 1999). Chronic activation of BKB_1-R is likely to be amplified by the accumulation of DBK at the site of inflammation (MARCEAU *et al.*, 1998). The up-regulation of the CPM (kininase I) may also account for the increasing endogenous level of desArg^{9}kinin metabolites and the BKB_1-R agonists in inflammation (SCHREMMER-DANNINGER *et al.*, 1998).

1.4.4.2.1. The BKB_1-R and circulation

The vasoconstrictor and vasodilator effects of the BKB_1-R have not yet demonstrated clearly their utility in pathological conditions. Hypothetically, ABBAS *et al.* (1998) proposed that the vasoconstrictor effect of the BKB_1-R in the umbilical human vein would allow the block of this vessel after delivery. *In vivo*, activation of the BKB_1-R or BKB_2-R induces hypotension, which allows supposing a role for these receptors in toxic shock, a cardiovascular condition that can be produced by injection of large doses of LPS. At low doses, the LPS induces the expression of the BKB_1-R without increasing the level of

the endogenous BKB_1-R agonist since injection of BKB_1-R antagonists does not change the basal blood pressure (DRAPEAU *et al.*, 1993). In contrast, after injection of high doses of LPS in rabbits, massive utilisation of kininogens and Hageman factor could be observed (ERDÖS & MIWA, 1968).

Using the LPS-induced septic shock model in piglets, SIEBECK *et al.* (1996) demonstrated that up-regulation of BKB_1-R was an important mechanism of host defence increasing the animals survival. In this model, BKB_2-R blockade attenuates LPS-induced pulmonary dysfunction and mortality, whereas additional BKB_1-R blockade seems to reverse these beneficial effects and increase the mortality rate. However, it has been recently shown that LPS-induced myocardial protection in rabbits subjected to ischaemia-reperfusion was not related to concomitant BKB_1-R induction (MAZENOT *et al.*, 2000).

The participation of BKB_1-R in blood pressure regulation was studied in BKB_2-R knockout mice with experimentally induced hypertension. It has been shown that in the absence of the BKB_2-R gene expression, the BKB_1-R can become up-regulated and assume some of the hemodynamic properties of the BKB_2-R (DUKA *et al.*, 2001). In the same study, it was shown that experimental manipulations to produce hypertension also induce up-regulation of the BKB_1-R, but not the BKB_2-R, in cardiac and renal tissues. Another study demonstrated that myocardial ischaemia-reperfusion could produce a global induction of functional BKB_1-R in the rabbit endothelium (MAZENOT *et al.*, 2001).

1.4.4.2.2. The BKB_1-R and oedema formation

The BKB_1-R appears to be involved in the development of local inflammatory responses (paw oedema/protein extravasation leading to joint swelling) in rat models of chronic arthritis (BHOOLA *et al.*, 2001). A pronounced up-regulation of BKB_1-R-mediated oedema has been reported in rats (CAMPOS *et al.*, 1998). The BKB_1-R-induced rat paw oedema was ascribed to the release of SP and CGRP from sensory C afferent fibres, serotonin from mast cells, and prostaglandin synthesis. This neurogenic response induced by the BKB_1-R was blocked by dexamethasone and cycloheximide, indicating the involvement of de novo protein synthesis in the up-regulation of the BKB_1-R (CAMPOS *et al.*, 1998). Further pharmacological evidence suggests that the manifestation of the BKB_1-R-mediated rat paw oedema, following intradermal injection of IL-1β and TNF-α, involves the activation of PKC, protein tyrosine kinase (PTK) or MAP-kinase and the NF-κB (CAMPOS *et al.*, 1999). A recent study also demonstrated that endotoxin induces a BKB_1-R-mediated oedema formation in non-human primate model (DEBLOIS & HORLICK, 2001).

1.4.4.2.3. The BKB_1-R and the cellular component of inflammatory response

The pro-inflammatory effects of BKB_1-R include promotion of blood-borne leukocyte trafficking, oedema and pain. In addition to the BKB_1-R, kallikreins and kininogens are found on the surface of circulating and synovial neutophils, which represents an effective way to deliver kinins at the site of inflammation (BHOOLA *et al.*, 2001). Activation of the BKB_1-R induces all three phases of leukocyte recruitment process; cell rolling, adhesion and emigration (McLEAN *et al.*, 2000). Furthermore, the BKB_1-R plays a role in the life span of

neutrophils at sites of inflammation as its presence is required for the maintenance of the apoptotic process in neutrophils (ARAÚJO *et al.*, 2001).

The BKB_1-R agonists act directly on sensory neurones to release SP and CGRP, which in turn influence neutrophil chemoattraction through endothelium expressing peptide receptors (AHLUWALIA & PERRETTI, 1999). The SP and CGRP induce rapid expression of vascular endothelial cell adhesion molecules (E-selectin, P-selectin and intercellular adhesion molecule-1), which play a primary role in the rolling and adhesion of circulating neutrophils (NAKAGAWA *et al.*, 1995). Alternatively, the BKB_1-R may activate indirectly sensory C afferent fibres through the release of prostaglandins, mast cell mediators and cytokines, especially interleukin-1 (DRAY & PERKINS, 1993; McLEAN *et al.*, 2000).

1.4.4.2.4. The BKB_1-R and peripheral inflammatory pain

Immunohistochemical studies have shown basal expression of the BKB_1-R in sensory ganglia as well as in central and peripheral nerve terminals of sensory neurones (Aδ and C-fibres) in the rat (Wotherspoon & Winter, 2000). However, BKB_1-R agonists did not affect nociception in normal rats or in acute models of inflammation (DRAY & PERKINS, 1997) and neither did they cause second messenger activation, neuropeptide release or electrophysiological events in sensory neurones under control or inflammatory conditions (DRAY *et al.*, 1992). Pharmacological antagonists of the BKB_1-R induced analgesia only in animal models of persistent inflammatory mechanical and thermal hyperalgesia (PERKINS *et al.*, 1993) or of persistent visceral pain (JAGGAR *et al.*, 1998). These results were explained by the induction of BKB_1-R on cells other than sensory neurones (macrophages, fibroblasts or endothelial cells) where they may be responsible for releasing mediators

(prostaglandins, cytokines and NO) that sensitize or activate the nociceptors (DRAY & PERKINS, 1997).

Furthermore, in a rat model of neuropathic hypersensitivity following peripheral nerve injury, analgesia was produced at 48 h and at 14 days post-injury by BKB_2-R antagonists, while BKB_1-R antagonists had analgesic effect only at 14 days after injury (LEVY & ZOCHODNE, 2000). The induction or up-regulation of the BKB_1-R and BKB_2-R in sensory neurones could also contribute the hyperalgesia following inflammatory pain (PETERSEN *et al.*, 1998). The direct effect of kinins on sensory neurones can be sensitized by the action of prostaglandins or other mediators released from other cells by the activation of either BK receptors.

The possibility that the BKB_1-R is involved in the physiological control of pain processes in the absence of inflammation cannot be excluded in species with constitutive BKB_1-R. PESQUERO *et al.* (2000) reported that, under normal non-inflamed conditions, the BKB_1-R-deficient mice proved to be hypoalgesic in behavioural tests of chemical and thermal nociception. With this exception, the BKB_1-R is mainly involved in persistent inflammatory pain and still its potential role in the control of acute pain in humans remains to be proven.

1.4.4.2.5. The BKB_1-R and diabetes

Autoimmune type 1 diabetes is the result of a breakdown of self-tolerance. It is associated with an over-production of cytokines, including IL-1β and TNF-α, which leads to T-cell-mediated pancreatic β-cell destruction (HUSSAIN *et al.*, 1996; RABINOVITCH & SUAREZ-PINZON, 1998; RABINOVITCH, 1998). These initial events are associated with an inflammatory reaction and the activation of an array of mediators including kinins.

The BKB_1-R, generally silent or absent in healthy states, is induced or activated under pathological conditions including type 1 diabetes, where the over-production of cytokines, the hyperglycemia and the oxidative stress are critical factors for its up-regulation (MARCEAU *et al.*, 1998; COUTURE *et al.*, 2001). Several mechanisms are involved in the up-regulation of the BKB_1-R in type 1 diabetes including the cytokines (IL-1β and TNF-α)-induced activation of the MAP-kinase and the NF-κB pathways (LARRIVÉE *et al.*, 1998; NI *et al.*, 1998; SCHANSTRA *et al.*, 1998; ZHOU *et al.*, 1998; SARDI *et al.*, 1998; CAMPOS *et al.* 1999). In addition, hyperglycemia and the resulting oxidative stress observed alongside diabetes can activate NF-κB (YERNENI *et al.*, 1999), which is known to induce the BKB_1-R (MARCEAU *et al.*, 1998). Therefore, both the over-production of cytokines and hyperglycemia could trigger the expression of the BKB_1-R through NF-κB in diabetes. Moreover, the long-term exposure of the BKB_1-R to its endogenous agonist DBK results in increased receptor expression (FAUSSNER *et al.*, 1999). Finally, the BKB_1-R was shown to be cross up-regulated by the BKB_2-R activation (via autocrine production of cytokines and activation of NF-κB) and/or BKB_2-R sensitization (PHAGOO *et al.*, 1999).

Accumulating evidence shows the up-regulation of the BKB_1-R subtype in streptozotocin (STZ)-diabetic animal models. It has been reported that the BKB_1-R is over-expressed in the stomach of STZ-diabetic mice since the sensitivity of the stomach fundus to DBK was substantially increased in these animals compared to control non-diabetic mice (PHENG *et al.*, 1997). Lung macrophages and fibroblasts from STZ-diabetic rats express the BKB_1-R and their activation leads to the release of cytokines (KOYAMA et al., 2000). In addition, the BKB_1-R is induced at the peripheral terminals of C-fibres and on the

endothelial cells in the lung of STZ-diabetic rats and its activation was shown to be associated with the release of SP (VIANNA *et al.*, 2003). The BKB_1-R subtype is also expressed in the kidney and spinal cord of STZ-treated mice (CLOUTIER & COUTURE 2000; MAGE *et al.*, 2002).

Furethermore, the BKB_1-R was recently shown to be up-regulated early in the retina of STZ-diabetic rats, 4 days and up to 21 days, following STZ injection (ABDOUH *et al.*, 2003). In the same study, selective BKB_1-R agonists evoked relaxation of the retinal vessels and the levels of the BKB_1-R binding sites remained steady and high over 21 days. Moreover, other studies coming from the same laboratory showed a significant increase in the level of the BKB_1-R mRNA expression in the spinal cord and brain of STZ-diabetic rats (2 and 7 days following the injection of STZ) and of its specific binding sites (2, 7 and 21 days following STZ injection) (ONGALI *et al.*, 2004).

Nevertheless, pharmacological evidence suggests that the BKB_1-R intervenes in the pathogenesis of STZ-induced diabetes in mice and that insulitis is a slow developing inflammatory reaction that is partly mediated through the activation of the BKB_1-R. ZUCCOLLO *et al.* (1996; 1999) demonstrated that BKB_1-R antagonists normalize glycemia and renal function. When administered with STZ, they reversed the elevation of blood glucose level and prevented the renal abnormalities, including increased urine volume and increased excretion of protein, nitrite and kallikrein. In addition, we proved that the BKB_1-R plays a determinant role in the increased vascular permeability associated with diabetes since BKB_1-R antagonists are able to inhibit the enhanced vascular permeability measured by extravasation of Evans Blue dye in several mouse tissues including the liver, pancreas, duodenum, ileum and kidney) (SIMARD *et al.*, 2001).

2. Diabetic neuropathy

Neuropathy is one of the major complications of diabetes (retinopathy, nephropathy, neuropathy, and atherosclerotic vascular diseases). Neuropathy is an irreversible problem that involves the majority of diabetic subjects and contributes to serious morbidity (affecting quality of life) in diabetic patients by causing loss of protective skin sensation in the feet (peripheral neuropathy; ulcerations; amputation), loss of normal function of the blood vessels in the skin and neuropathic pain.

Painful diabetic neuropathy (PDN), affecting 40-50 % of all diabetic patients at one stage of their life, results from an altered sensory excitability leading to hyperalgesia defined as augmented pain response to normally painful stimuli (this is often seen in response to heat) or allodynia defined as increased nociceptive responses to stimuli that are not normally perceived as painful stimuli (an example of this is wearing shoes or having bed sheets touching the feet) (SINGLETON *et al.*, 2001). Patients developing PDN are initially afflicted by tingling, paraesthesia and pain involving predominately the distal extremities (such as the lower limbs), in addition to persistent burning, stabbing down the legs which can be very debilitating especially at night (CALCUTT *et al.*, 1996; SAID, 1996) (Figure 14). The PDN remains impossible to prevent and very difficult to treat. Although several drugs (anticonvulsants, antidepressants) have been tried to ameliorate neuropathic pain in diabetic patients, their effectiveness is mitigated and associated with a number of side effects (BACKONJA & SERRA, 2004) and no other approaches beyond tight control of hyperglycemia (elevated glucose) have had a definite impact. Moreover, sensory polyneuropathy (poly = diffuse damage; the most common diabetic neuropathy) is NOT always a "late" complication of diabetes, but can occur early after the onset of diabetes and in diabetic children. However, in type 2 diabetes, neuropathies are frequently observed at diagnosis because of the relatively delayed recognition of the disease. Neuropathic pain may presage sensory loss, and subsequent loss of protective sensation contributes to foot ulceration and eventual amputation (Figure 15).

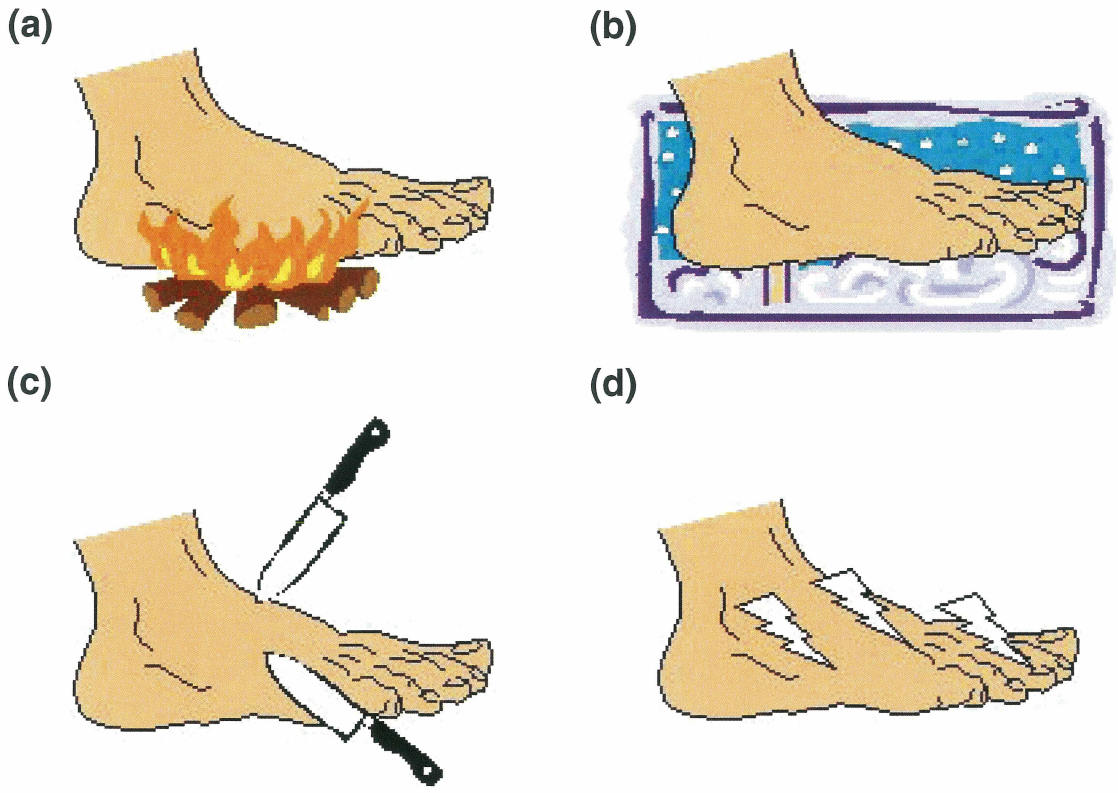

Figure 14. Symptoms of painful diabetic neuropathy (PDN). (a) Burning, feeling like the feet are on fire, **(b)** freezing, like the feet are on ice, although they feel warm to touch, **(c)** stabbing, like sharp knives and **(d)** lancinating, like electric shocks.

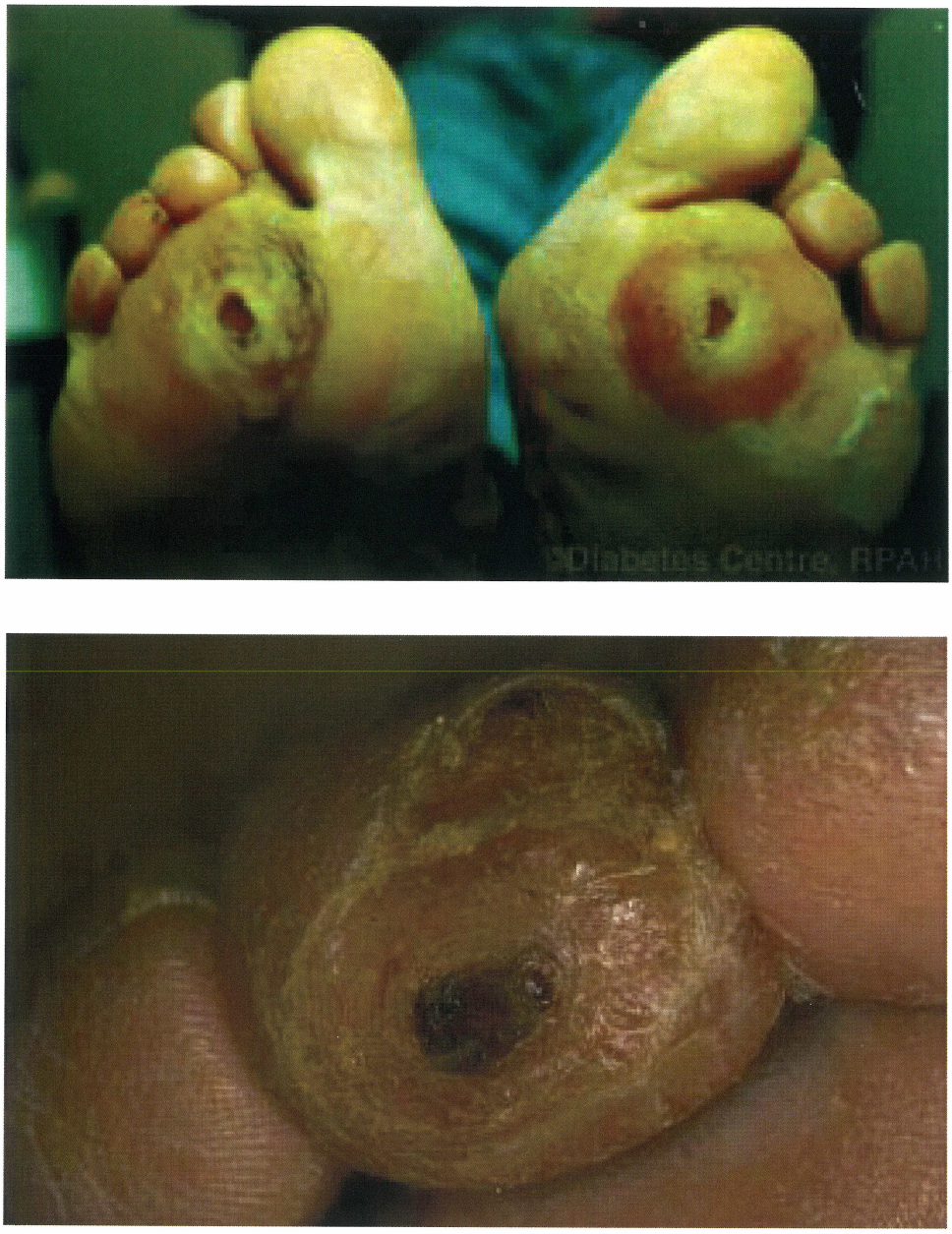

Figure 15. Two illustrations of diabetic sensory neuropathy of the foot. (Upper) a typical neuropathic ulcer at the bottom of the foot surrounded by callus and associated with good foot pulses (because the circulation is normal) and **(lower)** an affected foot having a callus and ulcer on the distal aspect of the right second toe. The patient was unaware of the ulcer.

2.1. Features and potential mechanisms of diabetic neuropathy

Many efforts were directed towards understanding how diabetes targets peripheral nerves: how and when they fail, and why they do not regenerate.

2.1.1. Neuropathic pain

Symptoms of painful sensory polyneuropathy, that develop in patients with mild diabetes or glucose intolerance, correlate with structural changes in cutaneous axons. Pain may be described as spontaneous electrical-like discharges from the feet, deep burning sensations or pricking discomfort. What local molecules either turn on or turn off pain at cutaneous axon terminals is unknown, nor is it appreciated how changes in their character might signal inappropriate pain discharges. There are a number of important possibilities to examine. Pathways and molecules promoting local pain include NO, nerve growth factor (NGF), BK, norepinephnine, neuropeptides (e.g. SP, CGRP), protons, glutamate and others (JORDT *et al.*, 2003; TOTH *et al.*, 2004). Recent work, however, has also suggested that local analgesic molecules may alternatively dampen pain. Some of these include local opioid peptides, galanin (an analgesic neuropeptide), and possibly neurotrophin-3 (NT-3; a relative of NGF). Receptors are known for and are expressed in sensory neurons (e.g. TrkA, p75 for NGF, B_1 and B_2 for bradykinin, CGRP-R1, NK-1 for SP, *N*-methyl-D-aspartate (NMDA) receptors for glutamate, transient receptor potential family (TRP) of cation channels, the most relevant of which are TRPV1 (previously known as VR1, or capsaicin receptors for heat and protons) and TRPM-8 previously known as CMR1 or menthol for cold (McKEMY *et al.*, 2002; TOTH *et al.*, 2004), but their expression on distal epidermal axons in the skin has had less

investigation. Changes in the distribution of sodium (Na^+) channels and N type Ca^{2+} channels that generate "ectopic" discharges (inappropriate generation of action potentials from axon hyperexcitability) develop following nerve injury but it is uncertain whether this occurs in distal axon terminals. In diabetes, an imbalance of pro-algesic and analgesic pathways, starting at abnormal cutaneous axon terminals, may promote neuropathic pain.

2.1.2. Loss of sensory neurons

Another mechanism related to diabetic neuropathy is the loss of sensory neurons. Human diabetic polyneuropathy is described as "stocking and glove" because the terminals of the longest axons that supply skin of the toes and fingers with sensation are the first involved. Normal skin nerve axons protect by warning of injury, contribute to balance through joint and pressure sensors, and control the caliber of cutaneous blood vessels that nourish the skin. By targeting sensory neurons and axons, diabetes leads to dysfunction, retraction and disappearance of skin axons (TOTH *et al.*, 2004) (Figures 16, 17 and 18). Alterations in diabetic sensory neurons from cell body to terminals have been recently demonstrated. The loss of sensory epidermal axons in a long term model of type 1 experimental diabetes in mice was shown, including alterations of the entire sensory neuron tree, and structural and electrophysiological features that better replicated human disease (KENNEDY & ZOCHODNE, 2005). In addition, CHERIAN *et al.* (1996) proved, in the rat model that first distal terminals are lost, followed later by cell body dropout. These findings are important because they identify an opportunity to rescue neurons before irretrievable loss.

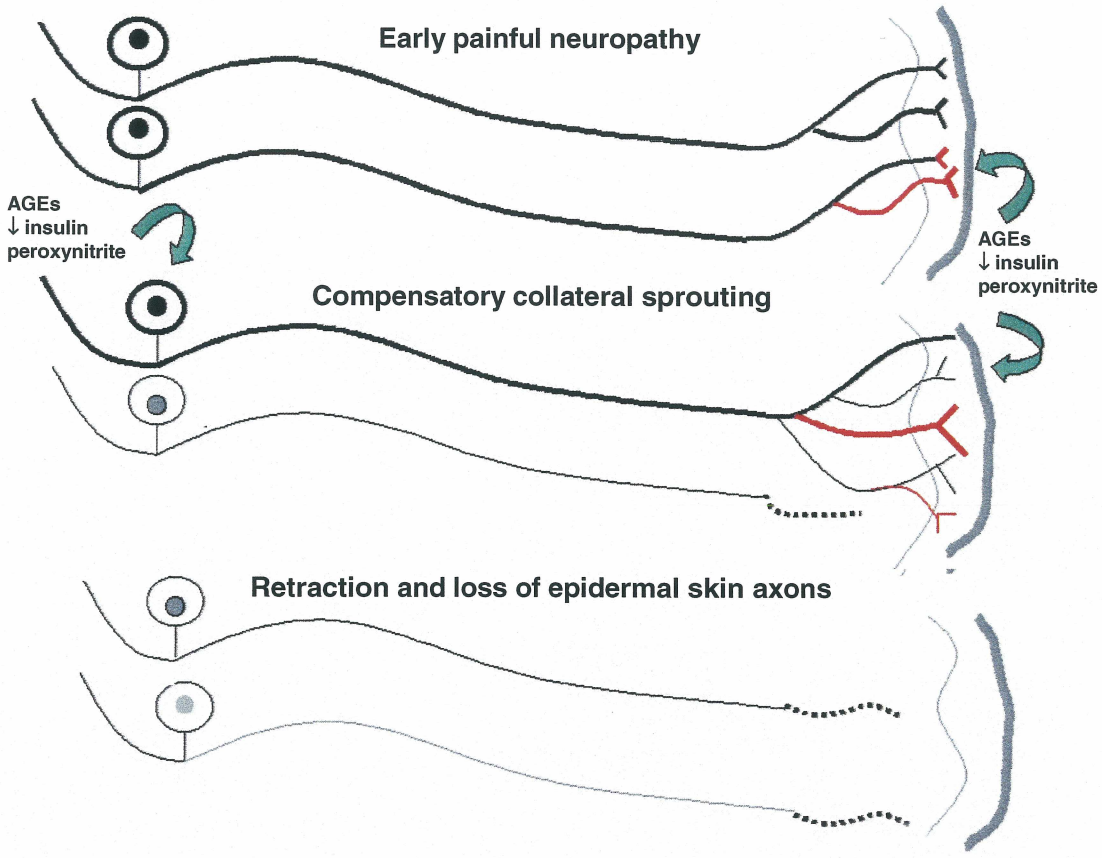

Figure 16. Epidermal skin axons loss in diabetes. Early phenotypic changes generate pain (red) when both nerve terminals and cell bodies are targeted by loss of insulin, advanced glycation end-products (AGEs) and peroxynitrite.

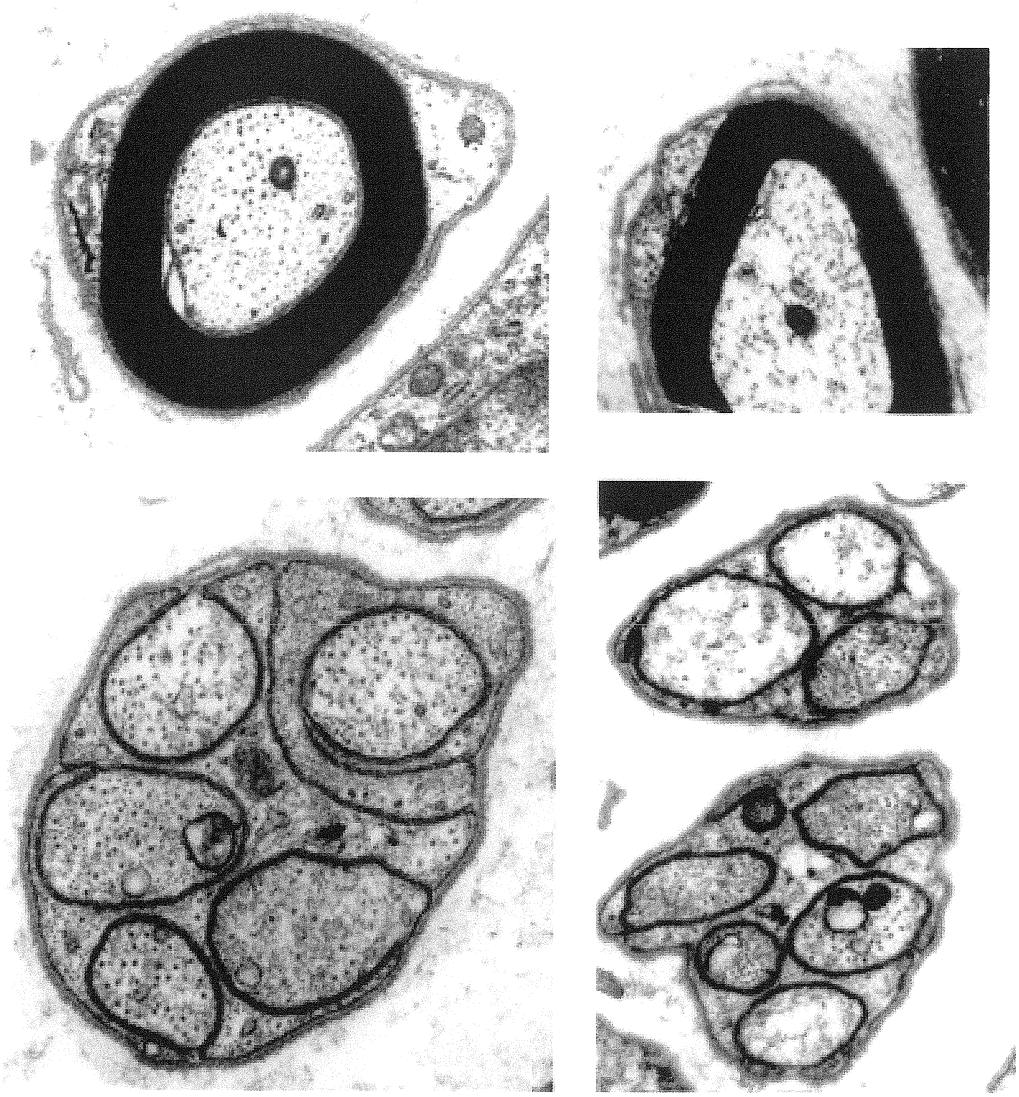

Figure 17. Comparison of the electron micrographs from small myelinated (upper) and un-myelinated (lower) fibres of diabetic (right) and non-diabetic (left) Sprague Dawley rats. Diabetic axons have fewer neurofilaments and microtubules. Note the atrophy of the diabetic unmyelinated fibres *(taken from SCOTT et al., 1999).*

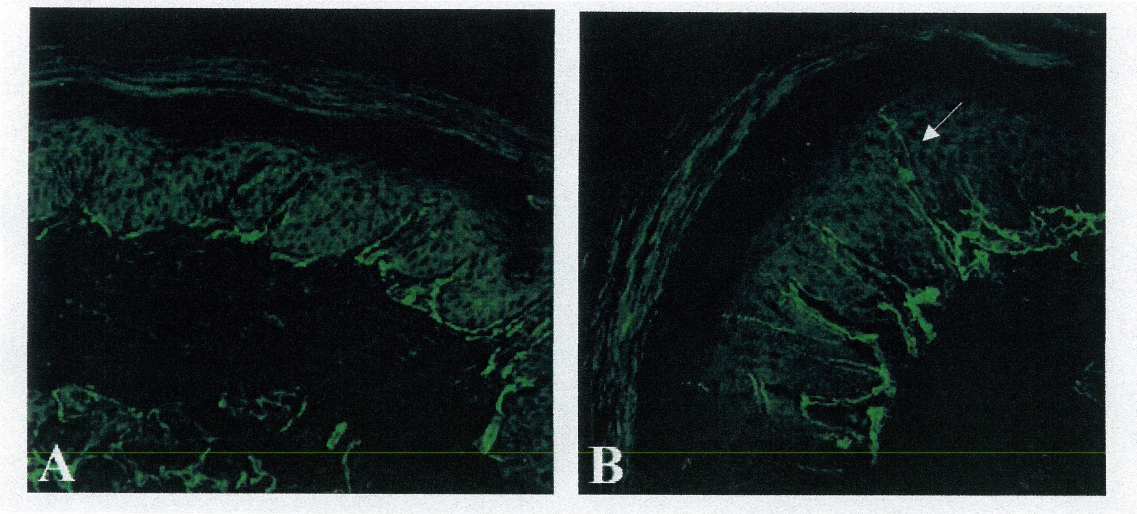

Figure 18. Significant loss of skin axons in streptozotocin (STZ)-diabetic mice. Sections were prepared from diabetic **(A)** or control non-diabetic mice **(B)**. Fine fluorescent axons entering the epidermis (arrow) are reduced in diabetic compared to non-diabetic mice *(taken from KENNEDY & ZOCHODNE, 2005)*.

Several mechanisms are implicated in sensory neuron damage in diabetes and have been extensively characterized (TOTH *et al.*, 2004):

Excessive accumulation of polyols (sugar alcohols) especially sorbitol, through the aldose reductase pathway in glial cells alters axon function (BORGHINI *et al.*, 1994). These changes are linked to depletions of nerve myo-inositol, changes in PKC subunits and dysfunction of nerve sodium/potassium adenosine triphosphatase (Na^+/K^+ATPase) (GUPTA *et al.*, 1992) that contribute to altered Na^+ content and channel properties of axons followed by slowing of nerve conduction velocity (CHERIAN *et al.*, 1996). Such slowing is a fundamental, widespread and early marker of diabetic polyneuropathy. Aldose reductase inhibitors (ARIs) or PKC inhibitors that interrupt this pathway have been suggested as therapy (ZENON *et al.*, 1990; ISHII *et al.*, 1996). How excessive polyol flux might induce degenerative neuronal disease, however, is unknown.

Free radical oxidative and nitrergic stress damages diabetic neurons (LOW *et al.*, 1997; LYONS & JENKINS, 1997). This pathway involves auto-oxidation of glucose and glycation products of glucose generate oxygen free radicals that include the hydroxyl radical, superoxide anion, hydrogen peroxide, singlet oxygen, NO and organic analogues. Antioxidant defences are lowered allowing targeting of lipids, DNA and proteins (WEST, 2000). The NO combines with superoxide anion to generate peroxynitrite, a highly potent oxidizing agent that nitrates protein tyrosines and can eventually lead to cell death (MALLOZZI *et al.*, 1997). It has been suggested that "nitrergic" (relating to NO and peroxynitrite) stress plays a significant role in the development of neuropathy. The NOS activity in ganglia and nerve are

increased, and there are footprints of peroxynitrite toxicity in ganglia (nitrotyrosine) (ZOCHODNE *et al.*, 2000; CHENG & ZOCHODNE, 2003).

Hyperglycemia and oxidative stress generate **advanced glycosylation end-products (AGEs)** that are thought to bind to the receptor of advanced glycation end-products (RAGE) to activate NF-κB, a potential mediator of cellular dysfunction (STERN *et al.*, 2002; WENDT *et al.*, 2003). Diabetic damage in non-neural tissues can be dramatically reduced by blocking RAGE activation. It has been also demonstrated that RAGE protein and mRNA are massively up-regulated in peripheral nerve and ganglia of STZ-diabetic mice (Figure 19) as well as in the kidney of obese diabetic (db/db) mice (model of type 2 diabetes) (Figure 20). There are early indicators that polyol flux, nitrergic stress and AGE-RAGE signaling are linked in important ways. For example, consumption of NO by AGEs in diabetes may contribute to loss of vasodilation (functional microangiopathy) in diabetic microvessels (BUCALA *et al.*, 1991; KIHARA & LOW, 1995).

In the face of diabetes, **alterations in trophic mechanisms** that support neurons are impaired (BREWSTER *et al.*, 1994). A large number of trophic factors that support neurons have been characterized including, for example, the neurotrophins [NGF, brain-derived neurotrophic factor (BDNF), NT-3, neurotrophin-4/5 (NT-4/5)], ciliary neurotrophic factor (CNTF), glial-cell-line-derived neurotrophic factor (GDNF), erythropoietin, insulin-like growth factors (IGFs) and insulin. Insulin emerges as an obvious candidate to consider in diabetes (ISHII, 1993; 1995).

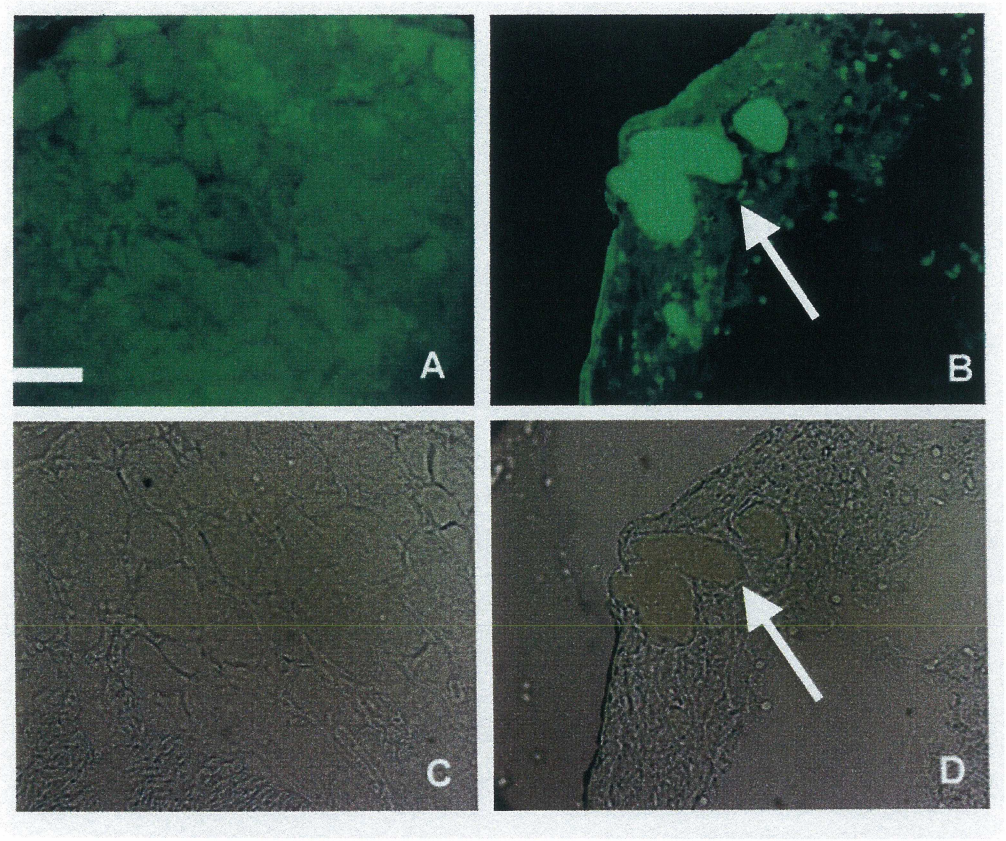

Figure 19. Heightened expression of the receptor of advanced glycation end-products (RAGE) in sensory neurons of STZ-diabetic male Swiss mice. Sections were prepared from control non-diabetic **(a)** or diabetic mice **(b)** and immunostaining was performed using anti-RAGE IgG. (c) and (d) are the same sections under light. The arrows show sensory neurons. Scale bar: 10 μm. *(unpublished work from ZOCHODNE laboratory, Department of Clinical Neurosciences, University of Calgary, AB, Canada)*.

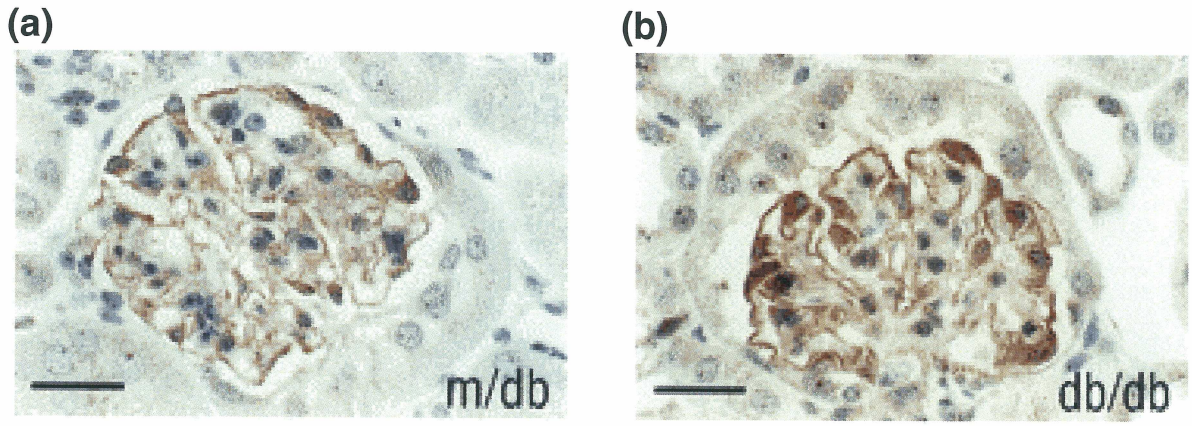

Figure 20. Expression of receptor of advanced glycation end-products (RAGE) antigen is enhanced in obese diabetic db/db kidney. Sections were prepared from non-diabetic m/db mice **(a)** or db/db mice **(b)** at 13 weeks of age and immunostaining was performed using anti-RAGE IgG. Scale bar: 16 μm *(taken from WENDT et al., 2003)*.

2.1.3. Blood vessels, skin and nerves

Micro-angiopathy, or damage to small nutrient blood vessels, may lead to ischemic damage of neurons and axons in diabetes (TOTH *et al.*, 2004) arising from endothelial damage and NO quenching during oxidative stress and polyol flux. These abnormalities are exacerbated by hyperviscosity, loss of red blood cell deformability, increased platelet aggregation alterations in local oxygen release, all likely contributing toward an eventual cascade of hypoxia and ischemia (TOTH *et al.*, 2004).

3. Hypothesis for the study

The kinin system regulates, in part, cardiovascular homeostasis, nociception and the acute phase of inflammation via the constitutive BKB_2-R subtype and participates in chronic inflammatory and nociceptive processes through its BKB_1-R subtype. Accumulating experimental evidence suggests that the BKB_1-R is up-regulated during the evolution of type 1 diabetes. The inducible BKB_1-R may play an important role in the development and/or progression of diabetic complications.

AIM OF THE WORK

The present project aimed at studying the development of hyperalgesia in rodent models of type 1 diabetes and highlighting the role of the inducible BKB_1-R receptor in this diabetic complication, through the use of selective BKB_1-R agonists and antagonists.

Several models of type 1 diabetes were used including the chemically-induced STZ models (CD-1 mice, C57BL/6 mice and Wistar rats) and the gene-prone spontaneous models (non-obese diabetic (NOD) mice and BioBreeding/Worcester (BB/Wor) rats).

Nociception was evaluated with two types of thermal pain tests; spinal (tail immersion and tail flick tests) and supra-spinal (hot plate and plantar stimulation) tests.

The selective BKB_1-R agonist, DBK, and its selective antagonists R-715 and R-954 were used for pharmacological characterization of the involvement of the BKB_1-R in diabetic hyperalgesia. In addition, a murine BKB_1-R-knockout genotype was used to confirm the pathophysiological role of the BKB_1-R in this complication.

The underlying mechanisms for the BKB_1-R-mediated diabetic hyperalgesia were also studied through several neurotransmitter systems including SP, NO and CGRP.

In addition, the profile of expression of the BKB_1-R in NOD mice was evaluated, as a function of age, in several target organs including the kidney and the spinal cord of diabetic NOD mice over 6 - 32 weeks of age.

In parallel, the effects of the potent BKB_1-R antagonist, R-954, were investigated on the increase in the cutaneous vascular permeability, which accounts partly for peripheral neuropathy, in diabetic animals.

RESULTS

Article 1

Role of bradykinin B_1 receptors in diabetes-induced hyperalgesia in streptozotocin-treated mice

Bichoy H. Gabra, Pierre Sirois

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Abstract

Insulin-dependent diabetes mellitus (type 1 diabetes) is an inflammatory autoimmune disease associated with vascular permeability changes leading to many complications including nephropathy, retinopathy, hypertension, hyperalgesia and neuropathy. The bradykinin B_1 receptor was recently found to be up-regulated during the development of diabetes and to be involved in its complications. Kinins are known to be important mediators of a variety of biological effects including cardiovascular homeostasis, inflammation and nociception.

In the present study, we studied the effect of the selective B_1 receptor agonist des-Arg^9-bradykinin and its specific antagonists R-715 (Ac-Lys-[D-ß Nal^7, Ile^8] des-Arg^9-bradykinin) and R-954 (Ac-Orn-[Oic^2, α-Me Phe^5, D-ß Nal^7, Ile^8] des-Arg^9-bradykinin) on diabetic hyperalgesia. Diabetes was induced in male CD-1 mice by injecting a single high dose of streptozotocin (200 mg kg^{-1}, i.p.) and the nociception was assessed using the hot plate and the tail flick tests, one week following the injection of streptozotocin. Our results showed that induction of diabetes by streptozotocin provoked a marked hyperalgesia in diabetic mice expressed as about 11 % decrease in hot plate reaction time and 26 % decrease in tail flick reaction time. Following acute administration of R-715 (200 - 800 μg kg^{-1}, i.p.), and R-954 (50 - 600 μg kg^{-1}, i.p.), this hyperalgesic activity was blocked and the hot plate and tail flick latencies of diabetic mice returned to normal values observed in control healthy mice. In addition, the acute administration of des-Arg^9-bradykinin (200 - 600 μg kg^{-1}, i.p.) significantly potentiated diabetes-induced hyperalgesia, an effect that was totally reversed by R-715 (1.6 - 2.4 mg kg^{-1}, i.p.) and R-954 (0.8 - 1.6 mg kg^{-1}, i.p.).

These results provide a major evidence for the implication of the bradykinin B_1 receptors in the development of hyperalgesia associated with diabetes and suggest a novel approach to the treatment of this diabetic complication using the bradykinin B_1 receptor antagonists.

Keywords: Insulin-dependent diabetes mellitus, hyperalgesia, kinins, bradykinin B_1 receptor, des-Arg^9-bradykinin, bradykinin B_1 receptor antagonists, R-715, R-954.

1. Introduction

Chronic inflammatory and cardiovascular diseases that often lead to pain are of increasing importance for health care in aging populations. Pain is a normal physiological protective mechanism to avoid tissue damage. In the periphery, it is signalled by fine C and Aδ afferent fibres that respond to noxious stimuli (mechanical, heat, cold, chemical). Indeed, all tissues, with the exception of the neuropil of the central nervous system (CNS), are innervated by such afferent fibres. However, pain is not a uniform sensation, and the quality of pain as well as the initiation of protective responses is determined by many factors within the spinal cord and in higher brain structures involved in the integration and modification of nociceptive signals (Dray, 1997).

When significant tissue damage occurs, pain is often more persistent and is associated with inflammation. In these circumstances, hyperalgesia and tenderness around the inflamed region occur. Activation and sensitization of peripheral nociceptors by chemical mediators produced by tissue injury and inflammation partially accounts for this. But in hyperalgesia, there is also facilitation of transmission at the level of the dorsal horn and the thalamus associated with changes in the central processing of pain signals, which allows signals generated by normally innocuous stimuli, such as gentle stroking, to be perceived as painful. In most cases, inflammation is a common and complex feature of clinical pain. The action of chemical mediators produced during inflammation is responsible for the multiplicity of events that occur, including hyperalgesia, alterations in cell phenotype, and the expression of new molecules (neurotransmitters, enzymes, ion channels, receptors) in the peripheral nervous system and the CNS (Levine et al., 1993; Dray, 1994)

Kinins have been known for some time to be important mediators implicated in a variety of biological effects including cardiovascular homeostasis, inflammation and nociception (for review see: Marceau et al., 1998; Calixto et al., 2000). They are probably the first mediators released in injured tissues from kininogens either by plasma kallikrein, which is activated early in the coagulation cascade, or tissue kallikrein, which is activated by proteases released at injured sites (Bhoola et al., 1992). Their production is critical for the initiation of pain and exaggeration of sensory signalling to produce hyperalgesia and allodynia. In addition, they promote many features of inflammation including an increase in blood flow and tissue oedema as well as the release of several mediators such as prostanoids and cytokines (Levine et al., 1993; Dray, 1994, 1995). Kinins mediate their biological effects by acting on two types of receptors, namely, B_1 and B_2. The B_2 receptors, which mediate many of the physiological effects of kinins, are constitutively expressed and involved in the acute phase of the inflammation and pain response. On the other hand, the B_1 receptors, usually absent in normal tissues, are highly induced and over-expressed during tissue injury and following treatment with inflammatory mediators like bacterial endotoxins and cytokines. They do not desensitize after agonist binding and participate in the chronic phase of the inflammation and pain response (Couture et al., 2001). The therapeutic value of intervention in the kallikrein-kinin system has not been fully explored. The known kinin receptors might be suitable pharmacological targets to treat chronic inflammatory and cardiovascular diseases and support new concepts of analgesic drug design through blockade of kinin receptors (Pesquero et al., 2000).

Diabetes mellitus is a term that describes a series of complex and chronic disorders characterized by symptomatic glucose intolerance due to defective insulin secretion, insulin

action or both. The chronic hyperglycemia of diabetes mellitus is associated with significant long-term sequelae, particularly damage, dysfunction and failure of various organs. The dysfunction of the vascular endothelium and the micro and macrovascular permeability changes lead to many diabetic complications including nephropathy, retinopathy, neuropathy, hypertension and hyperalgesia (Steil, 1999).

Experimental evidence suggests that diabetes up-regulates bradykinin B_1 receptors as a consequence of the over-production of cytokines and of the oxidative stress effect of hyperglycemia (Rabinovitch, 1998; Rabinovitch and Suarez-Pinzon, 1998; Yerneni et al., 1999). Other pharmacological studies suggest that the B_1 receptor intervenes in the pathogenesis of streptozotocin (STZ)-induced diabetes in mice as bradykinin B_1 receptor antagonists could normalize glycemia and renal function (Zucollo et al., 1996, 1999). In addition, it has been recently demonstrated that these antagonists were able to inhibit the increase in plasma extravasation associated with diabetic mice (Simard et al., 2002).

The STZ model is the most commonly used to study the cardiovascular and neuropathic complications of type 1 diabetes. STZ is an antibiotic extracted from *streptomyces acromogens*, which is selectively toxic for pancreatic islet β–cells. The decomposition products of STZ alter the cellular membrane proteins so that they are no longer recognized as self and thus initiating an autoimmune inflammatory process associated with cytokines (Wilson and Leiter, 1990; Lukić et al., 1998) resulting in the destruction of pancreatic β-islets. In addition, STZ can alter DNA in such a manner that a previously silent gene is expressed or a normal protein is altered by point mutation (Wilson and Leiter, 1990). The objective of the present study was to investigate the role of bradykinin B_1 receptors in hyperalgesia associated with the development of diabetes induced by STZ in mice. The effects of the

selective bradykinin B_1 receptor agonist des-Arg^9-bradykinin (Regoli et al., 1998, 2001) and its specific antagonists Ac-Lys-[D-ß Nal^7, Ile^8] des-Arg^9-bradykinin (R-715) and Ac-Orn-[Oic^2, α-Me Phe^5, D-ß Nal^7, Ile^8] des-Arg^9-bradykinin (R-954) (Neugebauer et al., 2002) were studied on the hyperalgesic response in diabetic mice.

2. Materials and methods

2.1. Animals

Male CD-1 mice weighing between 25-30 g (Charles River Breeding Laboratory, St. Constant, PQ, Canada) were used. The mice were housed four by cage with free access to food and water. They were maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10 %) with food and water available *ad libitum*. Animals were used only once in a given experiment. Experiments were conducted between 10:00 and 18:00 h. All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines and were approved by the Animal Care Committee of University of Sherbrooke.

2.2. Drugs

Streptozotocin (Pharmacia & Upjohn Inc., Mississauga, Ontario, Canada) was dissolved in saline at a pH of 4.5 and administered to mice i.p. The bradykinin-related peptides: des-Arg^{9}-bradykinin, R-715 and R-954 were synthesized by Dr. Witold Neugebauer in the Institute of Pharmacology of Sherbrooke, School of Medicine, University of Sherbrooke, Canada. They were dissolved in saline and administered to mice i.p.

2.3. Methods

2.3.1. Induction of type 1 diabetes

Insulin-dependent diabetes mellitus was induced in mice using STZ. Male CD-1 mice received a single high i.p. dose of STZ (200 mg kg^{-1}) (McEvoy et al., 1984). The induction

of diabetes was confirmed by measuring the blood glucose level 96 hours after STZ administration (Katovich et al., 1995; Chakir and Plante, 1996). Blood was withdrawn from the retro-orbital sinus of mice with a 50 µl heparinized capillary tube. Blood glucose levels were determined with an automatic analyzer (Glucometer Elite XL, Bayer Incorporation, Toronto, Ontario, Canada) using glucose oxidase / potassium ferricyanide reagents strips. The glucometer provides readings that are accurate within ± 1 mmol l^{-1} from 1.1 to 33.3 mmol l^{-1}. The diabetic animals used in our study had a blood glucose level higher than 20 mmol l^{-1} while the normal value is from 5 to 8 mmol l^{-1} (Chakir and Plante, 1996; Plante et al., 1996). The rate of induction of diabetes was 86 %.

2.3.2. Assessment of nociception

While nociception is defined as the normal electrophysiological response of peripheral sensory organs to noxious (tissue-damaging) stimuli, mediated by C and Aδ nociceptors, hyperalgesia is an exaggerated response to the same stimuli evoked by a hypersensitisation of peripheral nociceptors and a central facilitation of pain transmission at the level of the dorsal horn neurons and thalamus (Rang et al., 1999). Pain was measured in both healthy and diabetic mice using two types of thermal nociceptive tests:

2.3.2.1. The hot plate test. A hot plate test derived from that of Eddy and Leimbach (1953) was used. A plexiglass cylinder (20 × 14 cm) was used to confine the mouse to the anodized heated surface (275 × 263 mm) of the apparatus (IITC Hot Plate Analgesia Meter, Life Science, California, USA). The plate was adjusted to a temperature of 55 ± 0.5°C.

When the pain threshold is reached the animal starts to react by licking its hind paw or to jump, and the reaction time is recorded with a built-in timer, with a maximum cut-off time of 30 s to avoid tissue damage. Mice with latency value between 10-15 sec were selected.

2.3.2.2. The tail flick test. The tail flick test of D'amour and Smith (1941) modified for mice was used. The mice were habituated in a plexiglass cylindrical mouse restrainer (4 cm in diameter and 8 cm long), 15 min daily for one week before starting the experiments. To measure the latency of the tail flick response, mice were gently placed in the restrainer and the tail put in the tail groove of the apparatus (Model IITC 336 Paw/Tail Stimulator Analgesia Meter, Life Science, California, USA). The tail-flick response was elicited by applying radiant heat from a halogen bulb lamp (150 W) to the dorsal surface of the animal tail. The radiant light was focussed on a blackened spot in the mid region of the animal's tail (2-3 cm from the tip of the tail) and the latency between the application of the stimulation light and the flicking of the animal's tail was recorded. When the animal flicks its tail, its exposes a photocell in the apparatus immediately below the tail and the instrument is automatically stopped and the time is automatically recorded. A cut off time of 10 s was used to prevent blistering. The intensity of radiation was set at 40 to provide a pre-drug tail-flick response of 4-5 sec.

2.4. Experimental protocol

In both the hot plate and the tail flick tests, pre-treatment latencies were determined 3 times with an interval of 24 h starting 3 days before the injection of STZ or saline and the mean was calculated in order to obtain stable pre-drug response latency. On day 7 following the injection of STZ, the selective bradykinin B_1 receptor agonist des-Arg^9-bradykinin and/or

its specific antagonists R-715 and R-954 were given i.p. and the effect of their acute administration on nociception were determined at different time intervals. The mice were divided into the following groups: *(i)* control group, treated with saline; *(ii)* group treated with STZ (200 mg kg^{-1}, i.p., once); (iii) group treated with STZ + R-715 (200 - 800 μg kg^{-1}, i.p.); *(iv)* group treated with streptozotocin + R-954 (50 - 600 μg kg^{-1}, i.p.); *(v)* group treated with streptozotocin + des-Arg^9-bradykinin (200 - 600 μg kg^{-1}, i.p.); *(vi)* group treated with streptozotocin + des-Arg^9-bradykinin (400 μg kg^{-1}, i.p.) + R-715 (1.6 - 2.4 mg kg^{-1}, i.p.); *(vii)* group treated with streptozotocin + des-Arg^9-bradykinin (400 μg kg^{-1}, i.p.) + R-954 (0.8 - 1.6 mg kg^{-1}, i.p.); and *(viii, ix and x)* groups received only R-715 or R-954 or des-Arg^9-bradykinin, respectively. Each group was made of 6 - 10 mice.

The effect of the selected drugs on nociception was determined by converting the hot plate and tail flick responses from latencies into MPE (Maximum Percent Effect) according to the following equation (Bhargava and Zhao, 1996):

$$(\%\ \text{MPE}) = \frac{(\text{Post-treatment latency} - \text{Pre-treatment latency})}{(\text{Cut-off time} - \text{Pre-treatment latency})} \times 100$$

2.5. Statistical Analysis

Data are expressed as Mean (% MPE) ± S.E.M. and analysis of variance (ANOVA) followed by the "Student-Newman-Keuls Multiple Comparisons Test" were performed using GraphPad Instat, version 2.01 (GraphPad Software, San Diego, CA USA). $P < 0.05$ was considered significant. ID50, the dose that inhibit hyperalgesia in diabetic mice by 50% in the hot test plate and the tail flick tests, was estimated at time 20 min, which correspond to the maximal effect observed at each of the 5 doses used for both tested drugs. The ID50 values

were calculated using SigmaPlot, version 5.0 (SPSS Science, Chicago, IL USA) based on a curve-fit using GraphPad Prism (GraphPad Software) between the dose of the drug and % inhibition of hyperalgesic activity.

3. Results

3.1. Streptozotocin-induced hyperalgesia

Seven days following the injection of STZ, a marked hyperlagesia developed in diabetic mice. The MPE in the hot plate test was established at 0.76 ± 0.09 % and -11.51 ± 0.67% in control and STZ-diabetic mice, respectively (Fig. 1-A). The tail flick test also revealed a MPE of 0.53 ± 0.08% and -26.41 ± 0.90% in control and diabetic mice, respectively (Fig. 1-B). Both increased hyperalgesic activities were stable over time (60 min) (Fig. 2-A, 4-A).

3.2. The hot plate test

Administration (i.p.) of increasing doses of R-715 (100 - 800 μg kg^{-1}) or R-954 (50 - 600 μg kg^{-1}) did not affect the nociceptive threshold (baseline) in control healthy mice (data not shown). Conversely, R-715 produced an inhibition of the hyperalgesic activity observed in diabetic mice, which was dose- and time-dependent (Fig. 2-A, 2-B).

Maximal inhibition was observed after 20 min with all doses of R-715. Complete inhibition (99%, $P < 0.0001$; back to normal values in control mice) was reached after 20 min at a dose of 400 μg kg^{-1}, whereas lower dosages (100 and 200 μg kg^{-1}) at that same time did significantly inhibit (21 and 60%, respectively; $F = 143.49$, $P < 0.0001$) diabetes-mediated hyperalgesia. Inhibition by high doses (≥ 400) of R-715 was reduced by half at 40 min, and completely receded after 60 min (Fig. 2-A). Lower doses followed a similar course. The R-715' ID_{50} at the time of the maximal inhibition (20 min) was estimated at 172 ± 2 μg kg^{-1}.

To further support the role of the B_1 receptor in diabetes-induced hyperalgesia, des-Arg^9-bradykinin was exogenously administered (200, 400 and 600 μg kg^{-1}, i.p.) to both groups

of mice. In control mice, des-Arg^9-bradykinin did not induce significant changes in nociceptive response (data not shown). Conversely, the des-Arg^9-bradykinin potentiated by 34 to 98% ($F = 28.78$, $P < 0.0001$) the hyperalgesic response in STZ-treated mice. At all doses, the effect was maximal at 20 min post-injection and receded after 40 min. Co-administration of des-Arg^9-bradykinin, at the selected mid-dose of 400 μg kg^{-1}, and R-715, at selected doses of 1.6, 2.0 and 2.4 mg kg^{-1}, reversed the potentiating effect of des-Arg^9-bradykinin on STZ-induced hyperalgesia and produced a marked shift ($F = 151.50$, $P < 0.0001$) in the hot plate latencies to values equivalent to those recorded in the control healthy mice (Fig. 2-B).

The more potent and stable analogue to R-715, R-954, was also administered in the same model. R-954, at half the dose (200 μg kg^{-1}) of R-715, abolished STZ-mediated hyperalgesia after 20 min (Fig, 3-A). Lower dosages (50, 100 μg kg-1) still significantly ($F = 169.55$, $P < 0.0001$) inhibited the hyperalgesic activity by 33 and 61%, respectively, after 20 min (maximal inhibition), its effectiveness slowly decreasing but over a longer period of time (up to 50 min). With higher doses (> 200), the R-954 inhibitory potency was reduced by half at 45 min and completely receded after 60 min (Fig. 3-A). Lower doses followed a similar course. The R-954' ID_{50} at the time of the maximal inhibition (20 min) was estimated at 78 ± 3 μg kg^{-1}.

Once more, the co-administration of des-Arg^9-bradykinin and R-954 was assessed. While des-Arg^9-bradykinin raised the MPE values in STZ-mice (as mentioned above), smaller doses of R-954 (0.8, 1.2 and 1.6 mg kg^{-1}), compared to R-715, also abolished the hyperalgesic activity induced by exogenous des-Arg^9-bradykinin and returned the hot plate latencies almost

to normal values observed in control mice ($F = 205.07$, $P < 0.0001$) (Fig. 3-B). The maximal inhibitory effect was observed at 20 min and later decreased over time until 60 min.

3.3. The tail flick test

Administration of increasing doses of R-715 (< 800 μg kg^{-1}) or R-954 (< 600 μg kg^{-1}) had no significant effect on nociception in the tail flick test in non-diabetic mice (data not shown). Conversely, both drugs attenuated the hyperalgesic activity observed in diabetic mice in a dose- and time-dependent manner (Fig. 4-A, 4-B).

Maximal inhibition was observed after 10 min at either doses of R-715. The maximal inhibition (76%, $P < 0.0001$) was observed at a dose of 800 μg kg^{-1} whereas lower dosages (100, 200, 400 and 600 μg kg-1) at 10 min significantly inhibit diabetes-mediated hyperalgesia by 24, 50, 68 and 67%, respectively ($F = 121.89$, $P < 0.0001$; Fig. 4-A). The calculated ID_{50} at T = 10 min is 143 ± 4 mg kg^{-1}. The inhibition at all doses remained stable for an additional 10 min (up to 20 min). It decreased thereafter over time and activity ceased after 50 min (Fig. 4-A).

The administration of des-Arg^9-bradykinin (200, 400 or 600 μg kg-1) in control mice did not induce significant changes in the tail flick test (data not shown). In STZ-mice, the potentiating effect of des-Arg^9-bradykinin (25 to 48%) was maximal after 10 min at all three doses ($F = 341.20$, $P < 0.0001$) and receded after 20 min (Fig. 4-B). Co-administration of des-Arg^9-bradykinin, at the selected mid-dose of 400 μg kg-1, and R-715, at selected doses of 1.6 to 2.4 mg kg^{-1}, reversed by 27 to 57 % ($P < 0.0001$), the potentiating effect of des-Arg^9-bradykinin on STZ-induced hyperalgesia observed at T = 10 min (Fig. 4-B). The inhibitory effect of R-715 was more potent at 5 and 20 min post-injection since

des-Arg^9-bradykinin at these time points did not significantly potentiate the STZ-induced hyperalgesia (Fig. 4-B). Finally, the effect of all three doses of R-715 receded after 40 min (Fig. 4-B).

R-954 (200 mg kg^{-1} and above) significantly inhibited by 76% ($P < 0.0001$) the STZ-induced hyperalgesia after 10 min (Fig. 5-A). Lower dosages (50, 100 μg kg-1) still significantly ($F = 147.28$, $P < 0.0001$) inhibited the hyperalgesic activity by 28 and 51%, respectively, after 10 min. The inhibitory effect remained stable and receded after 20 min to ceased at 50 min (Fig. 5-A). The R-954' ID_{50} at the time of the maximal inhibition (10 min) was estimated at 72 ± 2 μg kg^{-1}.

Co-administration of des-Arg^9-bradykinin and R-954 exhibited the same profile of effects as observed with R-715 except that R-954 was more potent and lasted longer, up to 50 min (Fig. 5-B).

4. Discussion

Recent studies have demonstrated that the bradykinin B_1 receptors play a significant role in the pathogenesis of experimental diabetes and the development of its complications (Zucollo et al., 1996, 1999, Simard et al., 2002). However, there was no direct evidence for their involvement in the hyperalgesia induced in diabetic animals. In the present study, we showed that STZ-diabetic mice developed a marked hyperalgesia. This hyperalgesic effect was inhibited by single acute i.p. administration of the recently developed specific bradykinin B_1 receptor antagonists, R-715 (Regoli et al., 1998, 2001) and R-954 (Neugebauer et al., 2002). In addition, acute i.p. treatment with the selective bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, significantly potentiated the diabetes-induced hyperalgesia, an effect that was totally reversed by both R-715 and R-954.

The inducible bradykinin B_1 receptors were shown to participate in the chronic phase of the inflammatory and pain response (Dray and Perkins, 1993, 1997; Dray, 1997). They elicit persistent responses and signalling that are subject to very limited desensitization and receptor internalization with very low ligand dissociation (Couture et al., 2001). In addition, upon long-term agonist exposure, the B_1 receptor is up-regulated (Faussner et al., 1999). Chronic activation of B_1 receptors is likely to be amplified by the accumulation of des-Arg^9-bradykinin (the metabolite resulting from the degradation of bradykinin) at the site of inflammation because the half-life of des-Arg^9-bradykinin is 4- to 12-fold longer than that of bradykinin (Décarie et al., 1996a; Décarie et al., 1996b; Marceau et al., 1998). Up-regulation of carboxypeptidase M (kininase I, the enzyme responsible for the metabolism of bradykinin to des-Arg^9-bradykinin) may also account for the increasing endogenous level

of des-Arg^9-kinin metabolites and bradykinin B_1 receptor agonists in inflammation (Schremmer-Danninger et al., 1998). Moreover, a synergistic interaction appears to exist between bradykinin B_1 receptor ligands and interleukin-1β to enhance the expression of the bradykinin B_1 receptors (Phagoo et al., 1999). The induction of bradykinin B_1 receptor by cytokines is controlled by mitogen-activated protein kinase (MAP kinase) and by the nuclear transcriptional factor kappa B (NF-κB) (Campos et al., 1999).

Current evidence indicates that type-1 diabetes is due to an autoimmune response associated with over-production of cytokines, including interleukin-1β and tumour necrosis factor-α (TNF-α), that leads to the destruction of pancreatic islet β-cells (Rabinovitch, 1998; Rabinovitch and Suarez-Pinzon, 1998). These cytokines are believed to be implicated in the induction of the B_1 receptors (see above). In addition, hyperglycemia and the resulting oxidative stress can also activate the NF-κB (Yerneni et al., 1999), which is also involved in the induction of B_1 receptor expression (Marceau et al., 1998). Pharmacological evidence further suggests that the bradykinin B_1 receptor intervenes in the pathogenesis of STZ-induced diabetes in mice. To that effect, Zuccollo et al. (1996) demonstrated that bradykinin B_1 receptor antagonists normalize glycemia and renal function. When administered with STZ, they reversed the elevation of blood glucose level and prevented the renal abnormalities, including increased urine volume and increased excretion of protein, nitrite and kallikrein. Also, it has been established that B_1 receptors are over-expressed in the stomach of diabetic mice since the sensitivity of the stomach fundus to des-Arg^9-bradykinin was substantially increased in these animals (Pheng et al., 1997). Furthermore, it has been reported that bradykinin B_1 receptor plays a determinant role in the increased vascular permeability associated with diabetes as B_1 receptor antagonists could inhibit the enhanced permeability as

measured by extravasation of Evans Blue dye in several mouse tissues (liver, pancreas, duodenum, ileum, kidney) (Simard et al., 2002). More recently, the bradykinin B_1 receptor was reported to be induced in kidneys and spinal cord of rats treated three weeks earlier with STZ (Mage et al., 2002; Cloutier and Couture, 2000).

Regarding the pain process, recent immunohistochemical studies have shown the basal expression of B_1 receptors in sensory ganglia as well as in central and peripheral nerve terminals of sensory neurons (Aδ and C-fibres) in the rat (Wotherspoon and Winter, 2000). However, bradykinin B_1 receptors agonists neither affected nociception in normal rats nor in acute models of inflammation (Dray and Perkins, 1997) or caused second messenger activation, neuropeptide release or electrophysiological events in sensory neurons under control or inflammatory conditions (Dray et al., 1992). On the other hand, pharmacological antagonists of the bradykinin B_1 receptor induced analgesia only in animal models of persistent inflammatory mechanical and thermal hyperalgesia (Perkins et al., 1993, 1995; Rupniak et al., 1997; Poole et al., 1999; Bélichard et al., 2000) or of persistent visceral pain (Jaggar et al., 1998). Furthermore, in a rat model of neuropathic hypersensitivity following peripheral nerve injury, treatment with bradykinin B_1 receptor antagonists had an analgesic effect, 14 days after the injury (Levy and Zochodne, 2000). Nevertheless, it has been recently demonstrated that the hyperalgesia induced by Freund's adjuvant was reduced in bradykinin B_1 receptor-knockout mice (Ferreira et al., 2001). However, Pesquero et al. (2000) reported that under normal non-inflamed conditions, bradykinin B_1 receptor-deficient mice proved to be more resistant to pain in behavioural tests of chemical and thermal nociception. With this exception, B_1 receptors are mainly involved in persistent inflammatory pain and still the potential role of B_1 receptors in the control of acute pain needs further studies.

Moreover, Couture and Lindsey (2000) showed that activation of bradykinin B_1 receptors in STZ-pre-treated rats caused changes in the thermonociceptive threshold in the rat tail-flick test. The bradykinin B_1 receptor agonist des-Arg^9-bradykinin, administered intrathecally, did not affect the nociceptive threshold in control rats. However, the agonist induced a biphasic response in the animals, 24 h after treatment with STZ; an initial hyperalgesic response was noted 1 min after the injection, followed by a secondary antinociceptive effect 6 min after the B_1 agonist (Couture and Lindsey, 2000). The biphasic response was completely blocked by B_1 receptor antagonists, with the hyperalgesic effect also blocked by substance P antagonists, nitric oxide synthase (NOS)-inhibitors and cyclooxygenase-2 inhibitors, suggesting that central B_1 receptor activation is associated with the release of substance P and the production of nitric oxide and prostaglandins (Couture and Lindsey, 2000).

Our results showed that STZ-treated animals developed a well defined hyperalgesia that was evaluated in two types of thermal noxious tests, the hot plate test (supra-spinal) and the tail flick test (spinal). The diabetes-induced hyperalgesia was abolished by the specific bradykinin B_1 receptor antagonists, R-715 and R-954. In addition, the selective bradykinin B_1 receptor agonist, des-Arg^9-bradykinin significantly increased this hyperalgesic activity induced by diabetes, an effect that was almost completely reversed by R-715 and R-954. This exaggeration of sensory signaling and pain sensation could be attributed to the over-expression of the bradykinin B_1 receptors during the development of diabetes and to the increase in the endogenous level of des-Arg^9-bradykinin. Several mechanisms related to the up-regulation of bradykinin B_1 receptors could explain this diabetic hyperalgesia. The first potential mechanism that contributes to the hyperalgesia associated with diabetes is the

up-regulation of bradykinin B_1 receptors in sensory neurons (Petersen et al., 1998). The direct effect of kinins on sensory neurons to release substance P, calcitonin gene-related peptide (CGRP), neurokinin A and other nociceptive neurotransmitters, could be sensitized by the action of prostaglandins or other mediators released from other cells by the activation of kinin receptors. An alternative mechanism could be the induction of B_1 receptors on cells other than the sensory neurons (macrophages, fibroblasts or endothelial cells) which may be responsible for releasing mediators (prostaglandins, cytokines and nitric oxide) that sensitize or activate the nociceptors (Dray and Perkins, 1997). It has been also suggested by Walker et al. (1995) that stimulation of sympathetic nerves by kinins causes the release of neuropeptides, prostanoids, sympathetic transmitters or other mediators that sensitize nociceptive nerve terminals, leading to hyperalgesia. Furthermore the increase in vascular permeability induced by the bradykinin B_1 receptors is believed to enable the extravasation of blood constituents that might have a sensitizing effect on pain perception.

In conclusion, the bradykinin B_1 receptor participates in the chronic phase of inflammation and of somatic and visceral pain, and is likely to play a strategic role in diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes. Our results provide major evidence for the involvement of the bradykinin B_1 receptors in the development of inflammatory hyperalgesia associated with diabetes and suggest a novel approach to the treatment of this diabetic complication using bradykinin B_1 receptor antagonists.

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Legends for figures

Fig. 1. Evaluation of the nociceptive activity in control versus streptozotocin-diabetic mice using the hot plate (A) and tail flick (B) tests. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg kg^{-1}, i.p.). On day 7 following the induction of the disease, the hot plate test or the tail flick test was carried out. Data are expressed as mean % MPE ± S.E.M. (n = 10 - 14). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$.

Fig. 2. Effect of single acute administration of R-715 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-diabetic mice in the hot plate test. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg kg^{-1}, i.p.). On day 7 following the induction of the disease, mice were injected with R-715 (100 - 800 μg kg^{-1}, i.p.) **(A)**, des-Arg^9-bradykinin (DBK; 400 μg kg^{-1}, i.p.) **(B)** or a combination of DBK and R-715 (1.6 - 2.4 mg kg^{-1}, i.p.) **(B)**. The hot plate test was carried out at different time intervals (10 - 60 min) following injections. Data are expressed as mean % MPE ± S.E.M. (n = 7-12). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ significantly different from the STZ group at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; and $^{\$\$\$}$ significantly different from the STZ/DBK group at $P < 0.001$.

Fig. 3. Effect of acute administration of R-954 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-diabetic mice in the hot plate test. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg kg^{-1}, i.p.). On day 7 following the induction of the disease, mice were injected with R-954 (50 - 600 μg kg^{-1}, i.p.) **(A)**, des-Arg^9-bradykinin (DBK; 400 μg kg^{-1}, i.p.) **(B)** or a combination of DBK and R-954 (0.8 – 1.6 mg kg^{-1}, i.p.) **(B)**. The hot plate test was carried out at different time intervals (10 - 60 min) following injections. Data are expressed as mean % MPE ± S.E.M. (n = 7-12). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ significantly different from the STZ group at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; and $^{\$\$\$}$ significantly different from the STZ/DBK group at $P < 0.001$.

Fig. 4. Effect of single acute administration of R-715 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-treated diabetic mice in the tail flick test. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg kg^{-1}, i.p.). On day 7 following the induction of the disease, mice were injected with R-715 (100 - 800 μg kg^{-1}, i.p.) **(A)**, des-Arg^9-bradykinin (DBK; 400 μg kg^{-1}, i.p.) **(B)** or a combination of DBK and R-715 (1.6 - 2.4 mg kg^{-1}, i.p.) **(B)**. The tail flick test was carried out at different time intervals (10 - 60 min) following injections. Data are expressed as mean % MPE ± S.E.M. (n = 7-12). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; $^{\#}$ and $^{\#\#\#}$ significantly different

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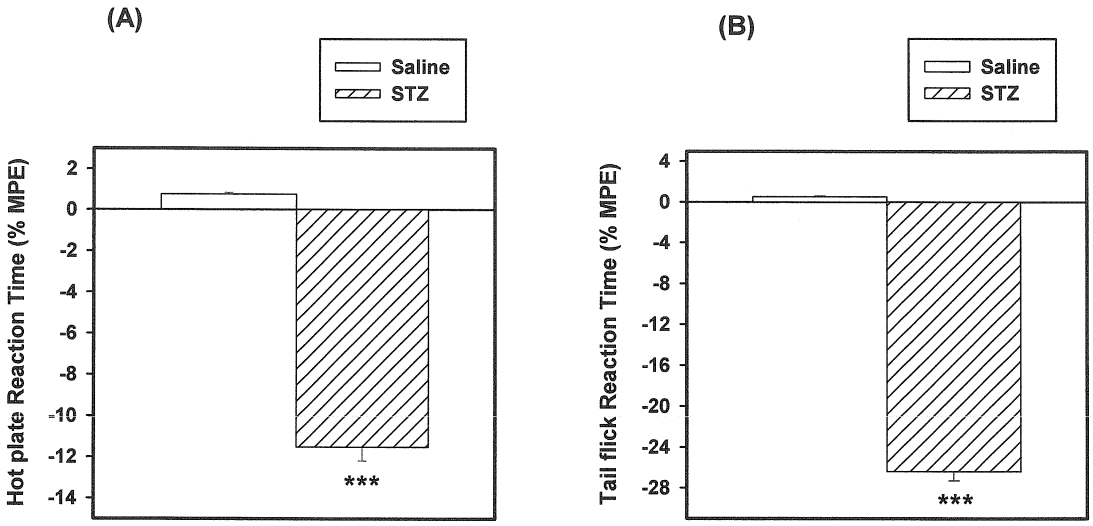
Fig. 5. Effect of single acute administration of R-954 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-treated diabetic mice using the tail flick test. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg kg^{-1}, i.p.). On day 7 following the induction of the disease, mice were injected with R-954 (50 - 600 μg kg^{-1}, i.p.) **(A)**, des-Arg^9-bradykinin (DBK; 400 μg kg^{-1}, i.p.) **(B)** or a combination of DBK and R-954 (0.8 – 1.6 mg kg^{-1}, i.p.) **(B)**. The tail flick test was carried out at different time intervals (10 - 60 min) following injections. Data are expressed as mean % MPE ± S.E.M. (n = 7-12). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ significantly different from the STZ group at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; $^{\$\$}$ and $^{\$\$\$}$ significantly different from the STZ/DBK group at $P < 0.01$ and $P < 0.001$, respectively.



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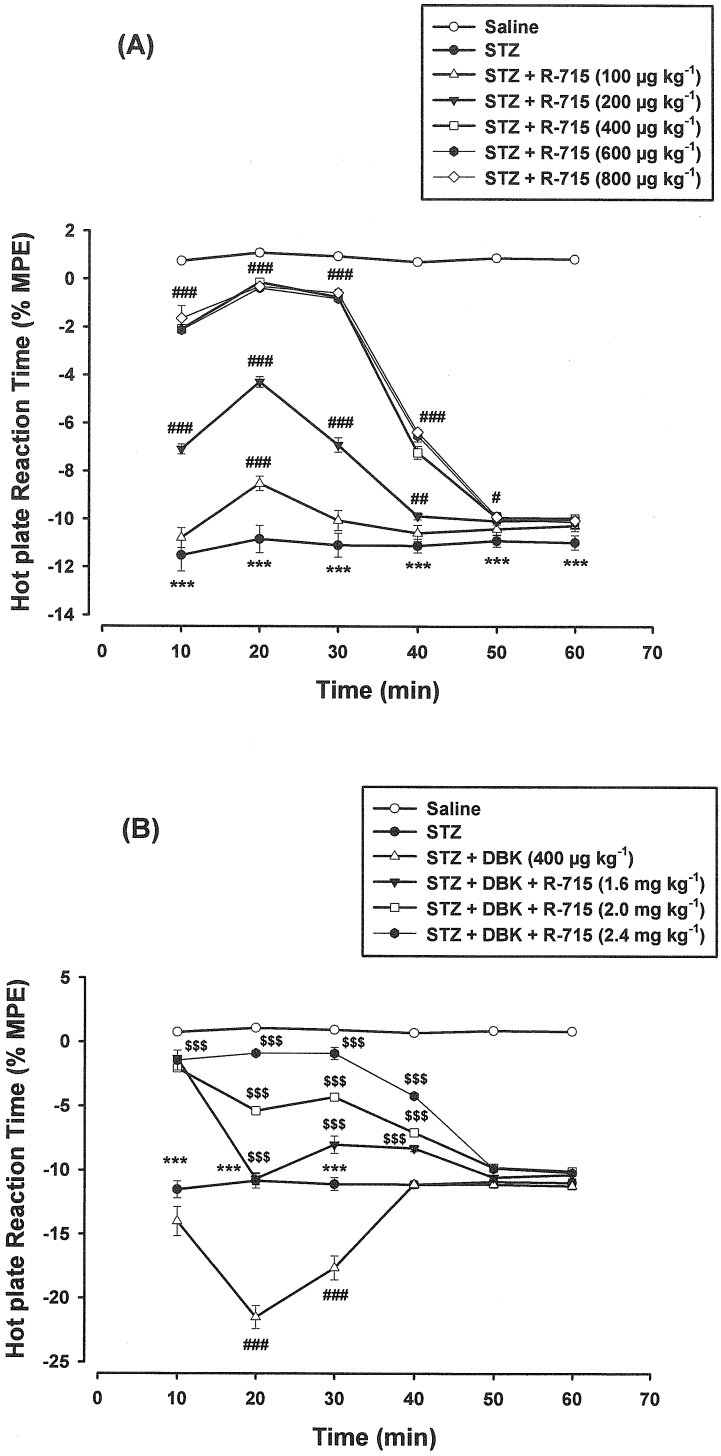
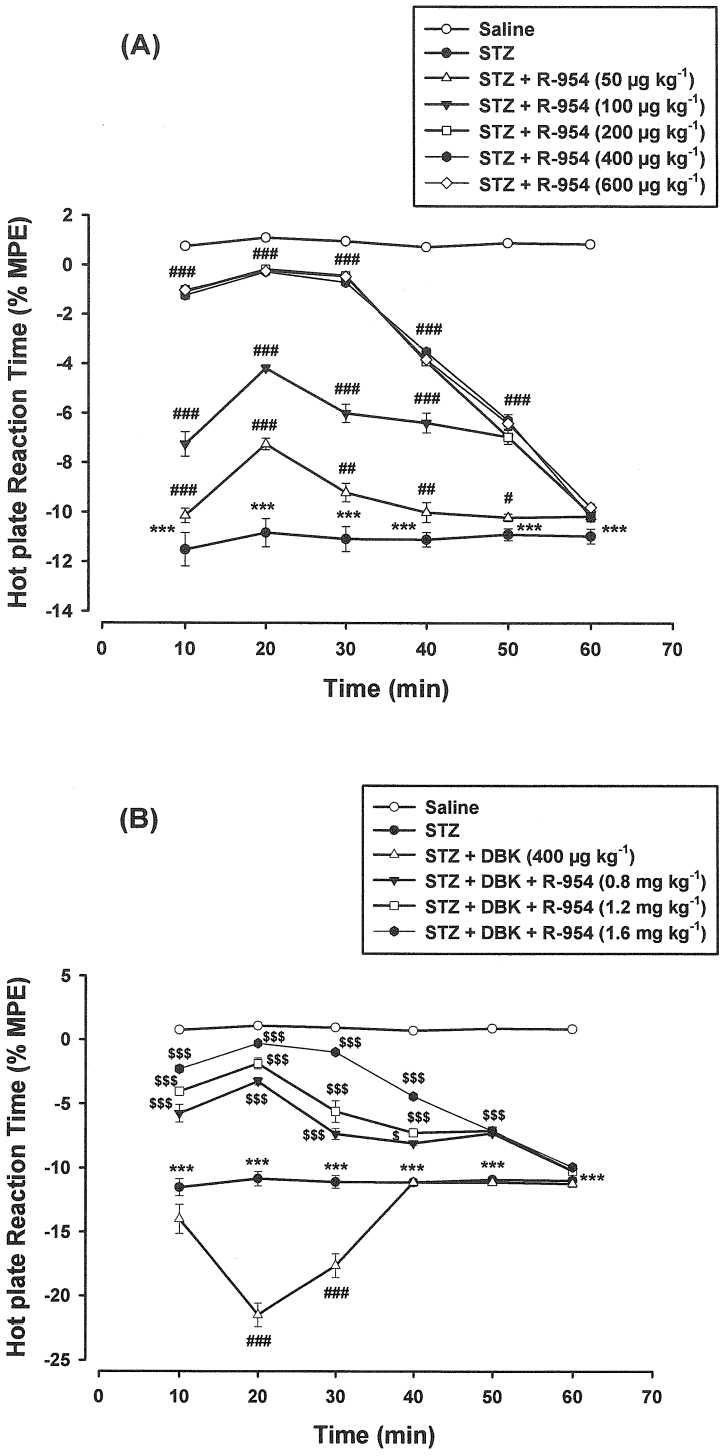


Fig. 2. Effect of single acute administration of R-715 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-diabetic mice in the hot plate test.



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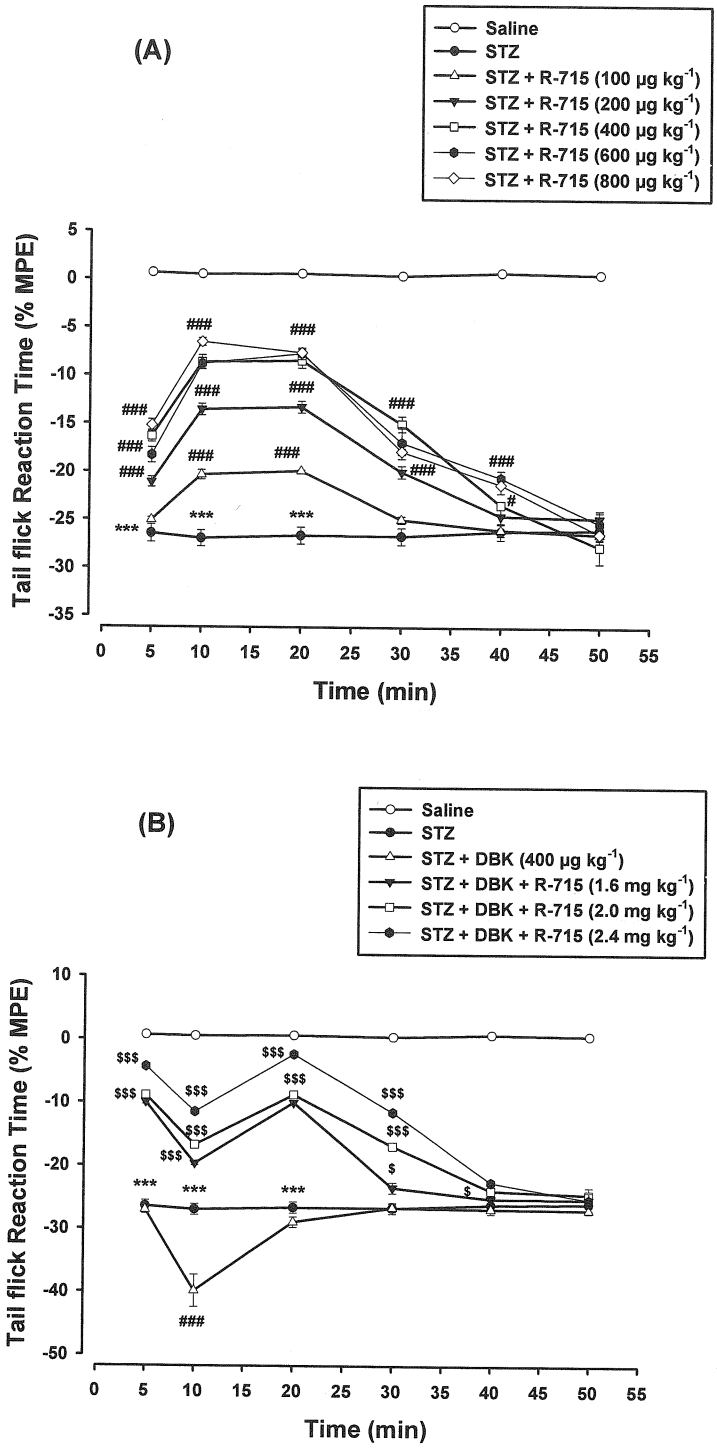

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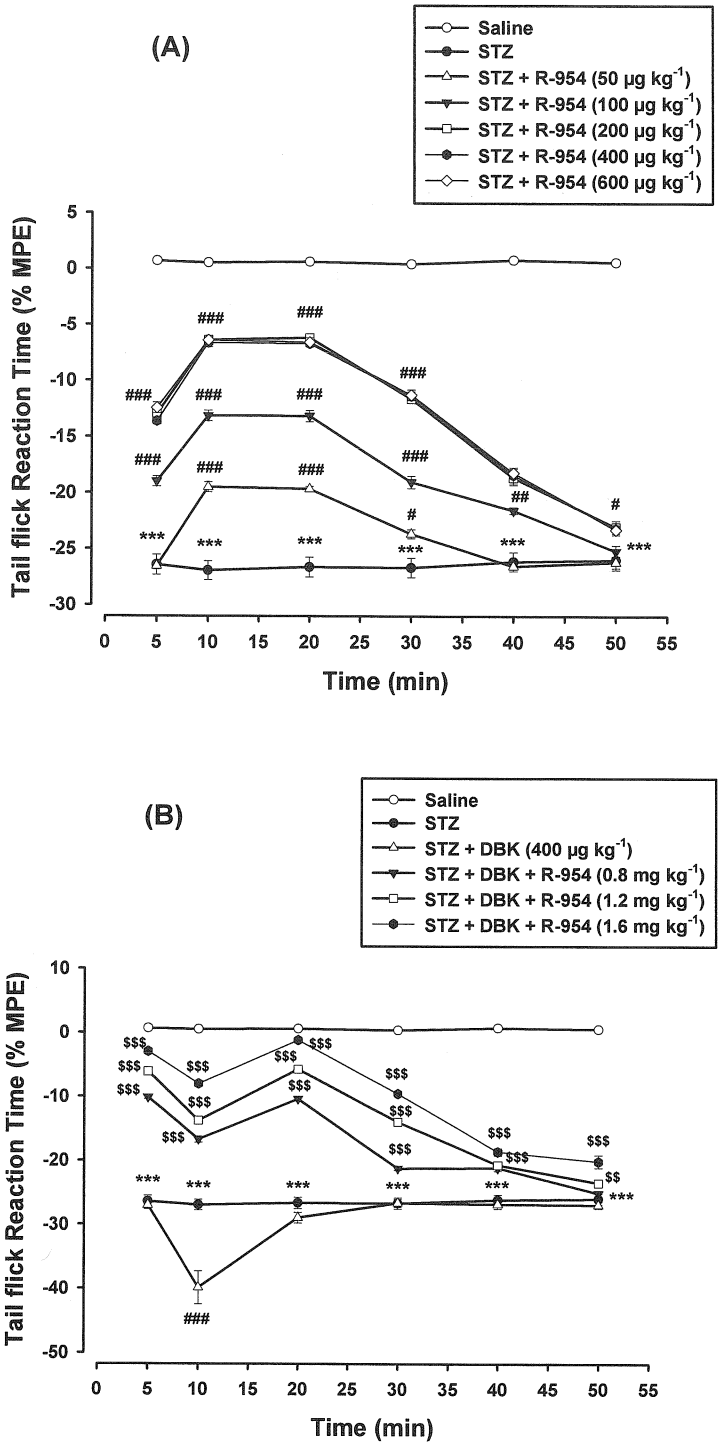

Fig. 5. Effect of single acute administration of R-954 (A) or its combined administration with des-Arg^9-bradykinin (B) on nociception in streptozotocin-treated diabetic mice using the tail flick test.

RESULTS

Article 2

Kinin B_1 receptor antagonists inhibit diabetes-induced hyperalgesia in mice

Bichoy H. Gabra, Pierre Sirois

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Abstract

Insulin-dependent diabetes mellitus (type 1 diabetes) is an inflammatory autoimmune disease associated with vascular permeability changes leading to many complications including nephropathy, retinopathy, neuropathy, hypertension and hyperalgesia. The bradykinin B_1 receptors (BKB_1-R) were recently found to be up-regulated alongside the development of type 1 diabetes and to be involved in its complications. Kinins are important mediators of a variety of biological effects including cardiovascular homeostasis, inflammation and nociception.

In the present study, we studied the effect of a selective BKB_1-R agonist desArg^9-BK (DBK) and two selective receptor antagonists, the R-715 (Ac-Lys-[D-ßNal^7, Ile^8]desArg^9-BK) and the R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ßNal^7, Ile^8]desArg^9-BK) on diabetic hyperalgesia. Type 1 diabetes was induced in male CD-1 mice via a single injection of streptozotocin (STZ, 200 mg/kg, i.p.), one week before the test. Nociception, a measure of hyperalgesia, was assessed using the plantar stimulation (Hargreaves) and the tail immersion tests.

The induction of type 1 diabetes provoked a significant hyperalgesic activity in diabetic mice, causing an 11 % decrease in plantar stimulation reaction time and 13 % decrease in tail immersion reaction time, compared to normal mice. Following acute administration of R-715 (100 - 600 µg/kg, i.p.), or R-954 (50 - 400 µg/kg, i.p.), the STZ-induced hyperalgesic activity was blocked in a dose-dependent manner and the hot plate and tail immersion latencies of diabetic mice returned to normal values observed in control healthy mice. In addition, the acute administration of DBK (400 µg/kg, i.p.) significantly potentiated diabetes-induced hyperalgesia, an effect that was totally reversed by R-715 (1.6 - 2.4 mg/kg, i.p.) and R-954 (0.8 - 1.2 mg/kg, i.p.). These results provide further evidence for the implication of the

BKB_1-R in type 1 diabetic hyperalgesia and suggest a novel approach in the treatment of this complication using the BKB_1-R antagonists.

Keywords: Insulin-dependent diabetes mellitus, hyperalgesia, kinins, bradykinin B_1 receptors, DBK, R-715, R-954.

1. Introduction

Pain is a normal physiological protective mechanism toward limiting tissue damage. In the periphery, it is signalled by fine C and Aδ afferent fibres that respond to various noxious stimuli (mechanical, heat, cold, chemical). Chronic inflammatory and cardiovascular diseases that often lead to pain are of increasing importance for health care (Dray, 1997). Inflammatory pain is usually associated with hyperalgesia (exaggerated or increased responsiveness to noxious stimuli) and tenderness around the inflamed region due to activation and sensitization of peripheral nociceptors by chemical mediators produced by tissue injury and inflammation (Dray, 1997). In addition, hyperalgesia involves facilitation of transmission at the level of the dorsal horn and the thalamus associated with changes in the central processing of pain signals, which allows signals generated by normally innocuous stimuli, such as gentle stroking, to be perceived as painful. In most cases, inflammation is a common and complex feature of clinical pain. The action of chemical mediators produced during inflammation is responsible for the multiplicity of events that occur, including hyperalgesia, alterations in cell phenotype, and the expression of new molecules (neurotransmitters, enzymes, ion channels, receptors) in the peripheral nervous system and the CNS (Levine et al., 1993; Dray, 1994)

Kinins are important mediators known to be implicated in many biological effects including cardiovascular homeostasis, inflammation and nociception (for review see: Marceau et al., 1998; Marceau and Bachvarov 1998; Calixto et al., 2000). They are believed to be the first mediators released in injured tissues from substrate kininogens either by plasma kallikrein, which is activated early in the coagulation cascade, or tissue kallikrein, which is activated by proteases released at injured sites (Bhoola et al., 1992). Their production

is critical for the initiation of pain and exaggeration of sensory signalling to produce hyperalgesia and allodynia. In addition, they promote many features of inflammation including an increase in blood flow and tissue oedema as well as the release of several mediators such as prostanoids and cytokines (Levine et al., 1993; Dray, 1994; 1995). Two types of receptors, namely, B_1 and B_2, mediate the biological effects of kinins. The B_2 receptor, which mediates many of the physiological effects of kinins, is constitutively expressed and reported to be responsible for the acute phase of the inflammation and pain response. On the other hand, the B_1 receptor, usually absent in normal tissues, is highly induced and over-expressed during tissue injury and following treatment with inflammatory mediators like bacterial endotoxins and cytokines. It does not desensitize after agonist binding and participates in the chronic phase of the inflammation and pain response (Couture et al., 2001).

Diabetes mellitus is a complex disorder characterized by symptomatic glucose intolerance due to defective insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes mellitus is associated with significant long-term sequelae, particularly damage, dysfunction and failure of various organs. The dysfunction of the vascular endothelium and the micro and macrovascular permeability changes lead to many diabetic complications including nephropathy, retinopathy, neuropathy, hypertension and hyperalgesia (Steil, 1999). Current experimental evidence suggests that diabetes up-regulates bradykinin B_1 receptors (BKB_1-R) as a consequence of the over-production of cytokines and of the oxidative stress effect of hyperglycemia (Rabinovitch, 1998; Yerneni et al., 1999). Other pharmacological studies reported that the BKB_1-R intervenes in the pathogenesis of streptozotocin-induced diabetes in mice since BKB_1-R antagonists could

normalize glycemia and renal function (Zucollo et al., 1996, 1999). In addition, it has been recently demonstrated that the same antagonists were able to inhibit the increase in plasma extravasation associated with diabetic mice (unpublished results). The Streptozotocin (STZ) model is the most commonly used to study the cardiovascular and neuropathic complications of type 1 diabetes. STZ is an antibiotic extracted from *streptomyces acromogens*, which is selectively toxic for pancreatic islet β–cells. The decomposition products of STZ alter the cellular membrane proteins so that they are no longer recognized as self and thus initiating an autoimmune inflammatory process associated with cytokines (Wilson and Leiter, 1990; Lukić et al., 1998) resulting in the destruction of pancreatic β-islets. In addition, STZ can alter DNA in such a manner that a previously silent gene is expressed or a normal protein is altered by point mutation (Wilson and Leiter, 1990).

Our objective is to investigate the role of BKB_1-R in the development of hyperalgesia associated with a STZ model of diabetic mice. The effect of the selective BKB_1-R agonist $desArg^9$-BK (DBK) (Regoli et al., 1998, 2001) and its specific antagonists R-715 (Regoli et al., 1998, 2001) and R-954 (Neugebauer et al., 2002) was studied on the hyperalgesic response in diabetic mice using the plantar stimulation (Hargreaves) and the tail immersion tests. We found that STZ-treated mice developed a well-defined hyperalgesia one week following the injection of STZ and this hyperalgesic effect was dose-dependently inhibited by the specicific BKB_1-R antagonists R-715 and R-954. On the other hand, the selective BKB_1-R agonist DBK significantly potentiated the diabetes-induced hyperalgesia, an effect that was reversed by co-administration of R-715 or R-954 with DBK.

2. Materials and methods

2.1. Animals

Male CD-1 mice of 5-6 weeks of age weighing between 25-30 g (Charles River Breeding Laboratory, St. Constant, PQ, Canada) were used. The mice were housed four by cage and maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10 %) with food and water available *at libitum*. Animals were used only once in a given experiment. Experiments were conducted between 10:00 and 18:00 h. All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines and were approved by the Animal Care Committee of the University of Sherbrooke.

2.2. Drugs

Streptozotocin (Pharmacia & Upjohn Incorporation, Mississauga, Ontario, Canada) was dissolved in saline at a pH of 4.5 and administered to mice i.p. The bradykinin (BK)-related peptides: desArg^9-BK (DBK), Ac-Lys-[D-ßNal^7, Ile^8]desArg^9-BK (R-715) and Ac-Orn-[Oic^2, α-MePhe^5, D-ßNal^7, Ile^8]desArg^9-BK (R-954) were synthesized by Dr. Witold Neugebauer in the Institute of Pharmacology of Sherbrooke, School of Medicine, University of Sherbrooke, Canada. They were dissolved in saline and administered to mice i.p.

2.3. Methods

2.3.1. Induction of type 1 diabetes

Insulin-dependent diabetes mellitus (IDDM) was induced in mice using STZ. Male CD-1 mice received a single high i.p. dose of STZ (200 mg/kg) (McEvoy et al., 1984). The induction of diabetes was confirmed by measuring the blood glucose level 96 h after STZ administration (Katovich et al., 1995; Chakir and Plante, 1996). Blood was drawn from the retro-orbital sinus of mice with a 50 µl heparinized capillary tube. Blood glucose levels were determined with an automatic analyzer (Glucometer Elite XL, Bayer Incorporation, Toronto, Ontario, Canada) using glucose oxidase/potassium ferricyanide reagents strips. The glucometer provides readings that are accurate within ± 1 mmol/l from 1.1 to 33.3 mmol/l. The diabetic animals used in our study had a blood glucose level higher than 20 mmol/l while the normal value is from 5 to 8 mmol/l (Chakir and Plante, 1996; Plante et al., 1996). The rate of induction of diabetes was 86 %.

2.3.2. Assessment of nociception

Nociception was measured in both healthy and diabetic mice using two types of thermal nociceptive tests:

The plantar stimulation (Hargreaves) test: The method of Hargreaves et al., (1988) was used. Mice were placed individually and trained in a plexiglass enclosures on top of a non-heated glass panel (Model IITC 336 Paw/Tail Stimulator Analgesia Meter, Life Science, California, USA) and left to acclimatize for 10 min daily for two days before starting the experiments. Light from a halogen bulb lamp (150 W) was delivered to the plantar surface

of one of the mouse hind-paws through the base of the glass panel and was focused using an aluminized parabolic mirror mounted on the light source. The time taken for the mouse to lift or lick its hind paw was noted. The intensity of the radiant heat (40) was selected in order to reach a basal latency of 8-10 sec, and to reduce the variability. A cut off time of 30 sec was used to avoid tissue damage. Each latency value was determined from two applications of the radiant heat stimulus, separated by 1-2 min intervals, and the mean of the two measures was taken.

The tail immersion test: The tail-immersion test was performed according to Coderre and Rollman (1983). The mouse was gently wrapped in a towel, held at a 45° angle to a thermostatically controlled water bath set at 52 ± 1°C. The latency between submersion of the tail and its removal from the water by the animal was recorded, with a maximum cut-off time of 10 sec to minimize tail skin tissue damage. Mice with latency value between 2.5-4.0 sec were selected.

2.4. Experimental protocol

In both the Hargreaves and the tail immersion tests, pre-treatment latencies were determined 3 times with an interval of 24 h starting 3 days before the injection of STZ or saline and the mean was calculated in order to obtain stable pre-drug response latency. On day 7 following the injection of STZ, the selective BKB_1-R agonist DBK and/or its specific antagonists R-715 and R-954 were given i.p. and the effect of their acute administration on nociception were determined at different time intervals. The mice were

divided into the following groups: *(i)* control group, treated with saline; *(ii)* group treated with STZ (200 mg/kg, i.p., once); (iii) group treated with STZ + R-715 (100-600 μg/kg, i.p.); *(iv)* group treated with STZ + R-954 (50-400 μg/kg, i.p.); *(v)* group treated with STZ + DBK (400 μg/kg, i.p.); *(vi)* group treated with STZ + DBK + R-715 (1.6-2.4 mg/kg, i.p.); *(vii)* group treated with STZ + DBK + R-954 (0.8-1.6 mg/kg, i.p.); and *(viii, ix and x)* groups received only R-715 or R-954 or DBK, respectively. Each dose of the BKB_1-R-related peptides was tested in a group of 6-10 mice.

The effect of the selected drugs on nociception was determined by converting the plantar stimulation and tail immersion responses from latencies into MPE (Maximum Percent Effect) according to the following equation (Bhargava and Zhao, 1996):

$$(\%\ \text{MPE}) = \frac{(\text{Post-treatment latency} - \text{Pre-treatment latency})}{(\text{Cut-off time} - \text{Pre-treatment latency})} \times 100$$

2.5. Statistical Analysis

Data are expressed as Mean (% MPE) ± S.E.M. and analysis of variance (ANOVA) followed by the "Student-Newman-Keuls Multiple Comparisons Test" were performed. $P < 0.05$ was considered significant.

3. Results

3.1. Induction of type 1 diabetes diabetes

Single treatment of mice with STZ (200 mg/kg, i.p.) produced a significant increase in blood glucose. The mean plasma glucose level, measured 96 h after STZ administration, was reported to be 29.8 ± 0.76 mmol/l in STZ-treted mice compared to 6.9 ± 0.23 mmol/l in control mice (Fig. 1).

3.2. STZ-mediated hyperalgesia

A significant hyperalgesic activity developed in diabetic mice, 7 days following the injection of STZ. The MPE in the Hargreaves test was -10.66 ± 0.42 % for the STZ-treated mice compared to 0.56 ± 0.11 % for the control group ($P < 0.0001$) (Fig. 2-A), whereas in the tail immersion test, diabetic mice showed a MPE of -12.98 ± 0.50 % versus 0.47 ± 0.05 % for the control ones ($P < 0.0001$) (Fig. 2-B).

3.3. Effect of R-715 and R-954 on diabetes-mediated hyperalgesia

The injection of increasing doses of the specific BKB_1-R antagonists R-715 (100-600 µg/kg) or R-954 (50-400 µg/kg) did not affect plantar stimulation or tail immersion latencies in the Hargreaves and tail immersion tests in control healthy mice (data not shown). However, acute i.p. administration of R-715 and R-954 produced a dose- and time-dependent inhibition of the hyperalgesic activity observed in diabetic mice in both tests (Fig. 3-A, 3-B, 3-C and 3-D).

In the Hargreaves test, the maximum effect was reached 20 min following the injection of all doses of R-715 and R-954. A 92 and 95 % inhibition of the hyperalgesic activity was

obtained after 20 min in diabetic mice with the doses of 400 and 600 μg/kg, respectively, whereas lower doses (100 and 200 μg/kg) significantly inhibited (39 and 62 %, respectively) diabetes-induced hyperalgesia at the same time ($F = 201.81$, $P < 0.0001$; Fig. 3-A). At 10 and 30 min following the administration of R-715, a similar but weaker inhibition of the diabetic hyperalgesia (compared to 20 min) was reported with all doses of R-715 ($F = 115.87$ and 166.27 after 10 and 30 min, respectively, $P < 0.0001$). Inhibition was significantly reduced at 40 min ($F = 4.29$, $P < 0.05$) and the effect receded after 50 min (Fig. 3-A).

The more potent and stable analogue to R-715, R-954 produced a maximum inhibition (92 %) of the hyperalgesic effect observed in diabetic mice at half the dose (200 μg/kg) of R-715 after 20 min (Fig. 3-B). Smaller doses (50 and 100 μg/kg) still significantly inhibited the diabetic hyperalgesia by 34 and 64 %, respectively, after 20 min ($F = 216.04$, $P < 0.0001$). The R-954 effectiveness slowly decreased but over a longer period of time (up to 50 min). With higher doses (200 and 400 μg/kg), the inhibitory effect of R-954 was reduced by 40 % at 40 min ($F = 156.04$, $P < 0.0001$) and by 65 % ($F = 16.48$, $P < 0.0001$) at 50 min and the effec disappeared after 60 min ($F = 1.72$, $P > 0.05$) (Fig. 3-B). Lower doses of R-954 followed a similar course.

Similarly, maximal increase in tail immersion reaction times was similarly observed after 20 min at either dose of R-715 and R-954. The maximal inhibition (95%, $P < 0.0001$) of diabetes-induced hyperlagesia in the tail immersion test was observed at doses of 400 and 600 μg/kg of R-715, whereas lower dosages (100 and 200 μg/kg) at 20 min significantly inhibit diabetes-mediated hyperlagesia by 36 and 60%, respectively ($F = 159.13$, $P < 0.0001$; Fig. 3-C).

At 10 and 30 min following the administration of R-715, a comparable but weaker inhibition of the hyperalgesic activity was obtained ($F = 34.32$ and 32.44, $P < 0.0001$ after 10 and 30 min, respectively), while the analgesic effect ceased after 40 min ($F = 0.46$ and $F = 0.08$, $P > 0.5$ after 40 and 50 min, respectively; Fig. 3-C).

On the other hand, the more potent BKB_1-R antagonist R-954 (200 mg/kg) significantly inhibited by 90% ($P < 0.0001$) streptozotocin-induced hyperalgesia after 20 min (Fig. 3-D). Lower dosages (50, 100 μg/kg) still significantly ($F = 135.90$, $P < 0.0001$) inhibited the hyperalgesic activity by 36 and 57%, respectively, after 20 min. The inhibitory effect of higher doses (200 and 400 μg/kg) decreased with time (68 and 37 % at 30 and 40 min, respectively) ($F = 86.54$ and 26.74, $P < 0.0001$ after 30 and 40 min, respectively), and the effect was completely abolished at 50 min (Fig. 3-D).

3.4. Effect of DBK on diabetes-mediated hyperalgesia

To further support the role of BKB_1-R in diabetes-induced hyperalgesia, DBK (400 μg/kg, i.p.), was exogenously administered to both diabetic and control mice. In non-diabetic mice, DBK did not induce significant changes in the plantar stimulation nor in the tail immersion latencies (data not shown). Conversely, DBK potentiated by 99% ($T = 20$ min, $P < 0.0001$) the hyperalgesic response in STZ-diabetic mice in the Hargreaves test (Fig. 4-A and 4-B). Co-administration of DBK with R-715 at selected doses of 1.6, 2.0 and 2.4 μg/kg, significantly and dose-dependently reversed the potentiating effect of DBK on STZ-induced hyperalgesia and produced a marked shift in the plantar stimulation latencies to values equivalent to those recorded in the control healthy mice ($F = 423.08$, $P < 0.0001$; Fig. 4-A). Likewise, co-administration of DBK with the more potent BKB1-R antagonist,

R-954 at lower doses (0.8, 1.2 and 1.6 μg/kg), compared to R-715, also abolished the hyperalgesic activity induced by exogenous DBK and returned the the plantar stimulation reactions times almost to normal values observed in control mice ($F = 415.83$, $P < 0.0001$; Fig. 4-B). With the two antagonists, the maximal inhibitory effect was observed at 20 min and later decreased over time. The effect persisted for 40 min with R-715 and for 50 min with R-954.

With regards to the tail immersion test, administration of DBK to STZ-mice, potentiated by 50% (T = 20 min, $P < 0.0001$) diabetes-mediated hyperalgesia (Fig. 4-C and 4-D). Once more, co-administration of DBK with R-715 at selected doses of 1.6, 2.0 and 2.4 μg/kg, reversed in a dose-dependent manner, the potentiating effect of DBK on STZ-induced hyperalgesia after 20 min (the time of maximum effect) ($F = 109.64$, $P < 0.0001$; Fig. 4-C). Finally, R-954 (0.8, 1.2 and 1.6 mg/kg) co-administered with DBK, showed a similar profile of effects as observed with R-715 (Fig. 4-D), except that R-954 was more potent and its effect lasted longer, up to 40 min versus 30 min for R-715.

4. Discussion

Recent studies have demonstrated that the BKB_1-R play a significant role in the pathogenesis of experimental diabetes and the development of its complications (Zucollo et al., 1996, 1999). However, there was no direct evidence for their involvement in the hyperalgesia induced in diabetic animals. In the current study, we demonstrated that mice treated with STZ developed a significant hyperalgesia. This hyperalgesic effect associated with diabetes, was inhibited by acute i.p. administration of the specific BKB_1-R antagonists, R-715 and R-954. In addition, acute i.p. treatment with the selective BKB_1-R agonist DBK significantly potentiated the diabetes-induced hyperalgesia, an effect that was totally inverted by both R-715 and R-954.

The inducible BKB_1-R is known to participate in the chronic phase of the inflammatory and pain response (Dray and Perkins, 1993, 1997; Dray, 1997). The BKB_1-R elicits persistent responses and signalling that are subject to very limited desensitization and receptor internalization with very low ligand dissociation (Couture et al., 2001). In addition, upon long-term agonist exposure, the BKB_1-R is up-regulated (Faussner et al., 1999). Chronic activation of BKB_1-R is likely to be amplified by the accumulation of DBK (the metabolite resulting from the degradation of BK) at the site of inflammation because the half-life of DBK is 4- to 12-fold longer than that of BK (Décarie et al., 1996a; Décarie et al., 1996b; Marceau et al., 1998). Up-regulation of carboxypeptidase M (kininase I, the enzyme responsible for the metabolism of BK to DBK) may also account for the increasing endogenous level of $desArg^9$-kinin metabolites and BKB_1-R agonists in inflammation (Schremmer-Danninger et al., 1998). Moreover a synergistic interaction appears to exist between BKB_1-R ligands and interleukin-1β to enhance the production of BKB_1-R (Phagoo

et al., 1999). The induction of BKB_1-R by cytokines (interleukin-1β and tumour necrosis factor-α; TNF-α) is controlled by mitogen-activated protein kinase (MAP kinase) and by the transcriptional nuclear factor KB (NF-κB) (Marceau et al., 1998; Campos et al., 1999).

Current evidence indicates that type 1 diabetes is due to an autoimmune response associated with over-production of cytokines, including interleukin-1β and TNF-α, that leads to the destruction of pancreatic islet β-cells (Rabinovitch, 1998). These cytokines are implicated in the induction of the BKB_1-R. In addition, hyperglycemia and the resulting diabetic oxidative stress can also activate the NF-κB (Yerneni et al., 1999). It has been also suggested that the BKB_1-R intervenes in the pathogenesis of STZ-induced diabetes in mice. Zuccollo et al. (1996) showed that BKB_1-R antagonists were able to normalize glycemia and renal function. When administered with STZ, they reversed the elevation of blood glucose level and prevented the renal abnormalities, including increased urine volume and increased excretion of protein, nitrite and kallikrein. Also, it has been shown that the BKB_1-R is over-expressed in the stomach of diabetic mice since the sensitivity of the stomach fundus to DBK was substantially increased in these animals (Pheng et al., 1997). Furthermore, it has been shown that BKB_1-R plays a determinant role in the increased vascular permeability associated with diabetes as BKB_1-R antagonists could inhibit the enhanced permeability as measured by extravasation of Evans Blue dye in several mouse tissues (liver, pancreas, duodenum, ileum, kidney) (unpublished results). Recently, the BKB_1-R was reported to be induced in both kidney and spinal cord of rats treated three weeks earlier with STZ (Mage et al., 2002; Cloutier and Couture, 2000).

Regarding the pain process, BKB_1-R agonists were not found to have an effect on nociception in normal rats or in acute models of inflammation (Dray and Perkins, 1997)

and neither did they cause second messenger activation, neuropeptide release or electrophysiological events in sensory neurons under control or inflammatory conditions (Dray et al., 1992). On the other hand, pharmacological antagonists of BKB_1-R induced analgesia only in animal models of persistent inflammatory mechanical and thermal hyperalgesia (Perkins et al., 1993, 1995; Rupniak et al., 1997; Poole et al., 1999; Bélichard et al., 2000) or of persistent visceral pain (Jaggar et al., 1998). Furthermore, in a rat model of neuropathic hypersensitivity following peripheral nerve injury, analgesia was produced 14 days post-injury by BKB_1-R antagonists (Levy and Zochodne, 2000). Nevertheless, it has been recently demonstrated that the hyperalgesic effect induced by Freund's adjuvant was significantly reduced in BKB_1-R knockout mice (Ferreira et al., 2001). However, Pesquero et al. (2000) reported that under normal non-inflamed conditions, BKB_1-R-deficient mice proved to be analgesic in behavioural tests of chemical and thermal nociception. With this exception, BKB_1-R are mainly involved in persistent inflammatory pain and still the potential role of BKB_1-R in the control of acute pain remains to be proven.

Several mechanisms, related to the up-regulation of BKB_1-R, could explain this phenomenon of diabetes-mediated hyperalgesia. The first potential mechanism that contributes to the hyperalgesia associated with diabetes is the up-regulation of BKB_1-R in sensory neurons (Petersen et al., 1998). Kinins act directly on sensory neurons to release substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A and other nociceptive neurotransmitters. This effect could be sensitized by the action of prostaglandins or other mediators released from other cells by the activation of kinin receptors. Another mechanism is based on the induction of BKB_1-R on cells other than the sensory neurons (macrophages, fibroblasts or endothelial cells) where they might be responsible for releasing mediators

(prostaglandins, cytokines and nitric oxide) that sensitize or activate the nociceptors (Dray and Perkins, 1997). In addition, it has been proposed that the stimulation of sympathetic nerves by DBK, might be impliceted in the development of hyperalgesia due to the release of neuropeptides, prostanoids, sympathetic transmitters or other mediators that sensitize nociceptive nerve terminals, (Walker et al., 1995). Finally, the increase in vascular permeability induced by BKB_1-R is believed to enable the extravasation of blood constituents that might have a sensitizing effect on pain perception.

In conclusion, the BKB_1-R participate in the chronic phase of inflammation and of somatic and visceral pain, and is likely to play a strategic role in diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes. The therapeutic value of intervention in the kallikrein-kinin system has not been fully explored. The known kinin receptors might be suitable pharmacological targets to treat chronic inflammatory and cardiovascular diseases and support new concepts of analgesic drug design through blockade of kinin receptors. Our results provide major evidence for the involvement of BKB_1-R in the development of inflammatory hyperalgesia associated with diabetes and suggest a novel approach in the treatment of this diabetic complication using the BKB_1-R antagonists.

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Legends for figures

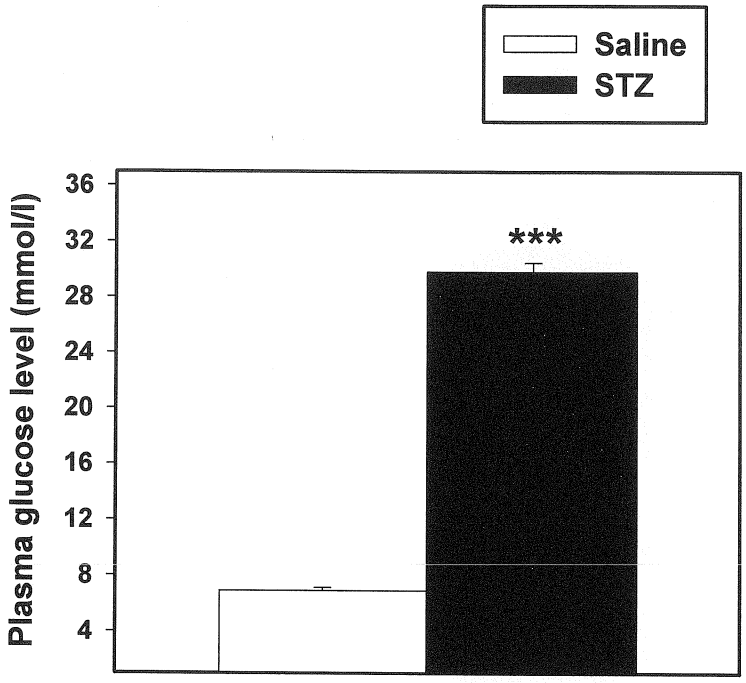
Fig. 1. Plasma glucose level in control versus streptozotocin-diabetic mice. CD-1 mice received single injection of either saline or streptozotocin (STZ; 200 mg/kg, i.p.). 96 h later, a blood sample was drawn from the retro-orbital sinus of mice and the plasma glucose level was determined using an automatic analyzer. Data are expressed as mean mmol/l ± S.E.M. (n = 60). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$.

Fig. 2. Assessment of nociception in control versus streptozotocin-diabetic mice using the Hargreaves (A) and tail immersion (B) tests. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg/kg, i.p.). On day 7 following the induction of the disease, the Hargreaves (plantar stimulation) test or the tail immersion test was carried out. Data are expressed as mean % MPE ± S.E.M. (n = 10-14). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$.

Fig. 3. Effect of acute administration of R-715 (A, C) or R-954 (B, D) on nociception in STZ-diabetic mice in the Hargreaves (A, B) and tail immersion (C, D) tests. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg/kg, i.p.). On day 7 following the induction of the disease, mice were given a single acute injection of R-715 (100 - 600 μg/kg, i.p.) or R-954 (50 - 400 μg/kg, i.p.). The Hargreaves (plantar stimulation) and tail immersion tests were carried out at different time intervals following R-715 or R-954 injection. Data are expressed as mean % MPE ± S.E.M. (n = 6-10). MPE = Maximum Percent Effect. n = number

of animals. *** significantly different from the saline group at $P < 0.001$; # and ### significantly different from the STZ group at $P < 0.05$ and $P < 0.001$, respectively.

Fig. 4. Effect of combined administration of DBK with R-715 (A, C) or R-954 (B, D) on nociception in STZ-diabetic mice in the Hargreaves (A, B) and the tail immersion (C, D) tests. Diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg/kg, i.p.). On day 7 following the induction of the disease, mice were given a single acute injection of DBK (400 μg/kg, i.p.) or a combination of DBK with R-715 (100 - 600 μg/kg, i.p.) or with R-954 (50 – 400 μg/kg, i.p.). The Hargreaves (plantar stimulation) and the tail immersion tests were carried out at different time intervals following injections. Data are expressed as mean % MPE ± S.E.M. (n = 6-10). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; #, ## and ### significantly different from the STZ group at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; $$ and $$$ significantly different from the STZ/DBK group at $P < 0.01$ and $P < 0.001$, respectively.



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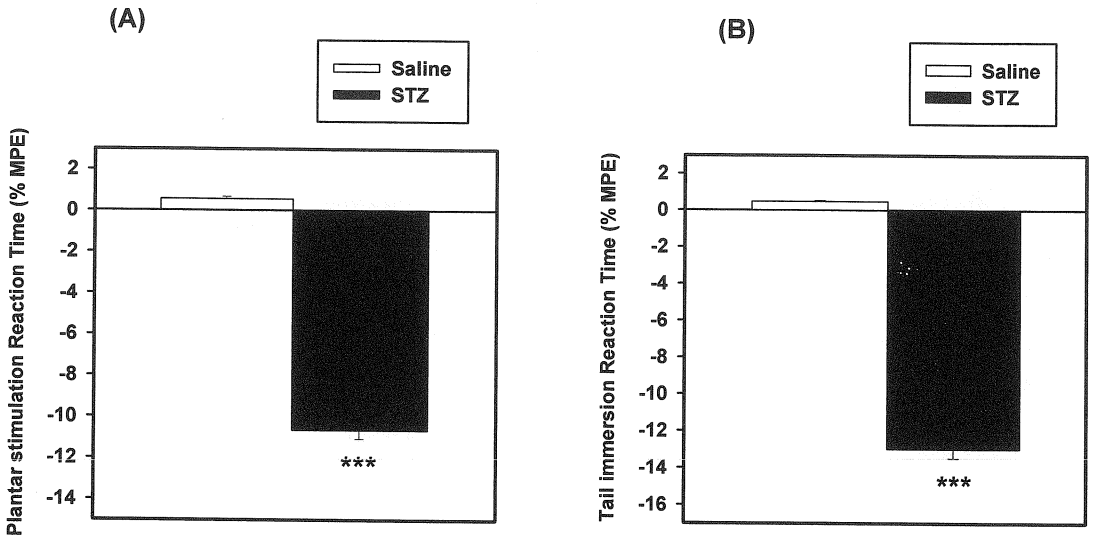

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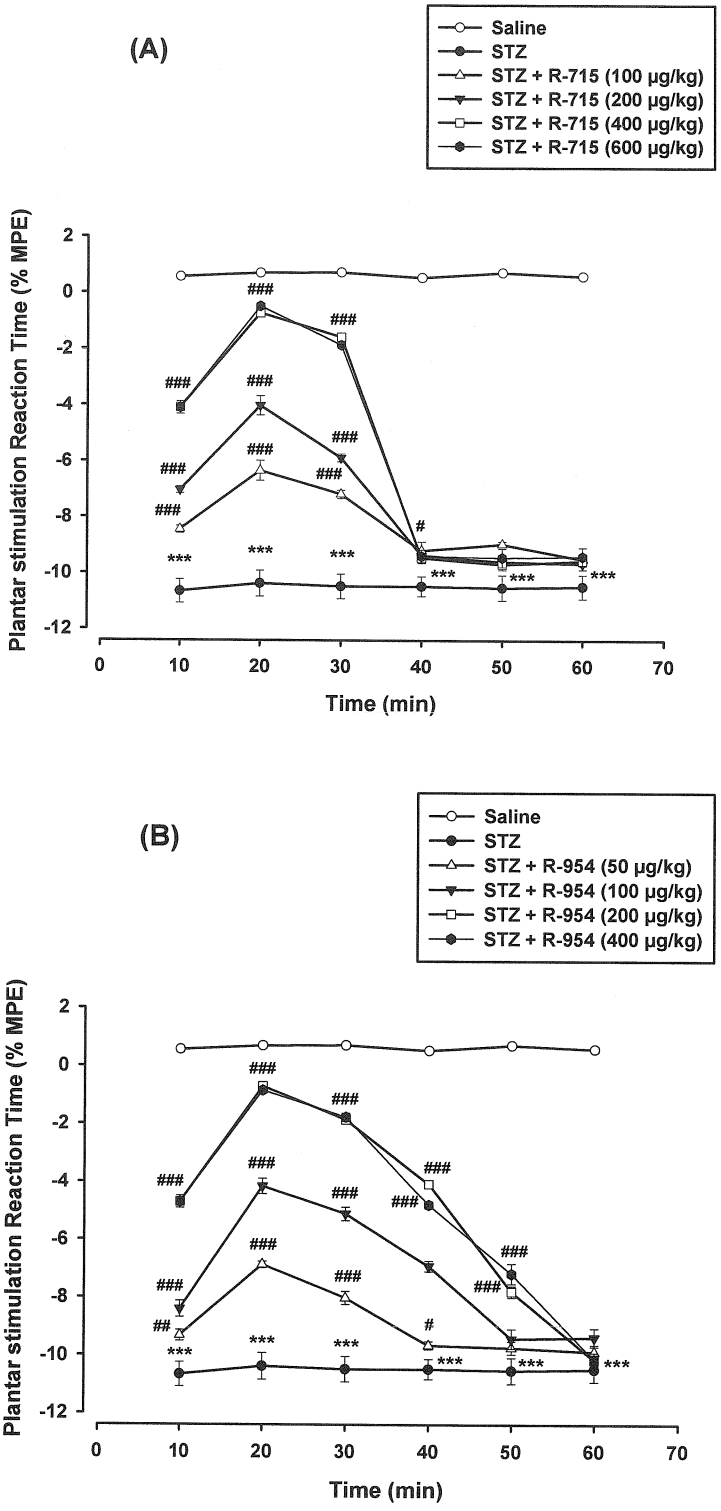

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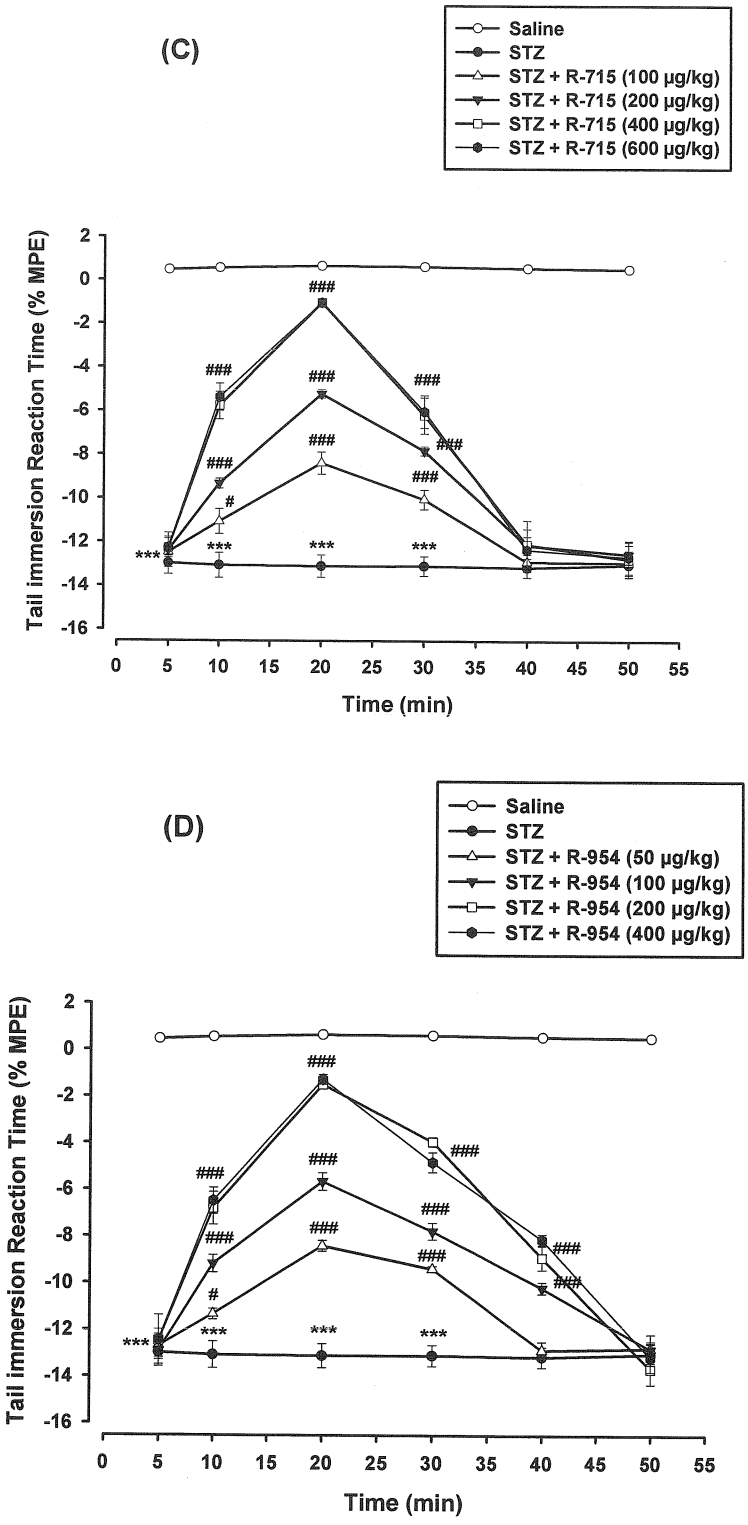

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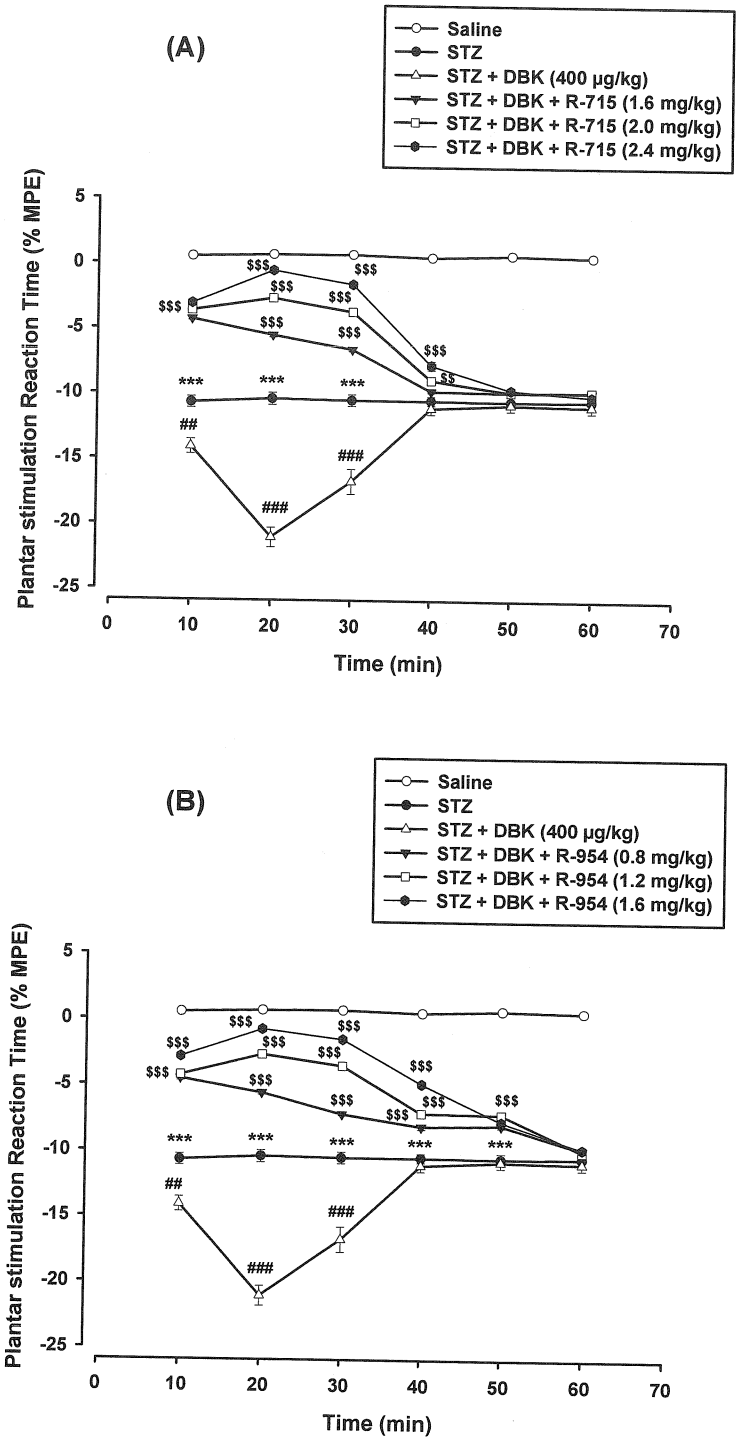

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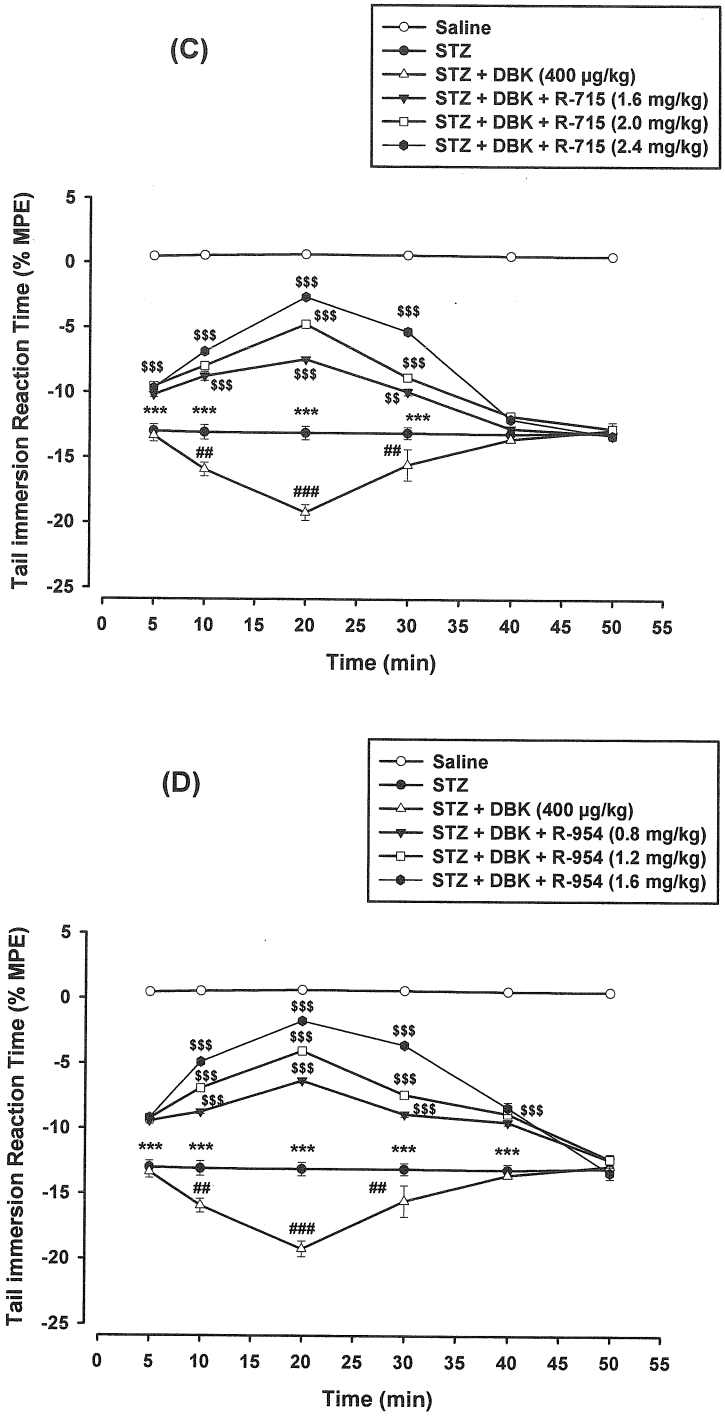

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RESULTS

Article 3

Beneficial effect of chronic treatment with the selective bradykinin B_1 receptor antagonists, R-715 and R-954, in attenuating streptozotocin-diabetic thermal hyperalgesia in mice

Bichoy H. Gabra, Pierre Sirois

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Abstract

Kinins are important mediators of cardiovascular homeostasis, inflammation and nociception. Bradykinin (BK) B_1 receptors (BKB_1-R) are over-expressed in pathological conditions including diabetes, and were reported to play a role in hyperglycemia, renal abnormalities, and altered vascular permeability associated with type 1 diabetes. Recent studies from our laboratory demonstrated that BKB_1-R are implicated in streptozotocin (STZ)-diabetes-mediated hyperalgesia, since acute administration of the selective BKB_1-R significantly and dose-dependently inhibited such hyperalgesic activity. In the present study, we examined the effect of chronic treatment of STZ-diabetic mice with the selective BKB_1-R agonist desArg^{9}bradykinin (DBK) and two specific antagonists R-715 and R-954, on diabetic hyperalgesia. Diabetes was induced in male CD-1 mice by injecting a single high dose of STZ (200 mg/kg, i.p.) and nociception was assessed using the hot plate, plantar stimulation, tail immersion and tail flick tests. Drugs were injected i.p. twice daily for 7 days, starting 4 days after STZ. We showed that chronically administered R-715 (400 μg/kg) and R-954 (200 μg/kg), significantly attenuated the hyperalgesic effect developed in STZ-diabetic mice as measured by the four thermal nociceptive tests. Further, chronic treatment with DBK (400 μg/kg) produced a marked potentiation of the hyperalgesic activity, an effect that was reversed by both R-715 and R-954. The results from this chronic study confirm a pivotal role of the BKB_1-R in the development of STZ-diabetic hyperalgesia and suggest a novel approach to the treatment of this short-term diabetic complication using BKB_1-R antagonists.

Keywords: Insulin-dependent diabetes mellitus; hyperalgesia; kinins; bradykinin B_1 receptor; desArg^{9}bradykinin; bradykinin B_1 receptor antagonists; R-715; R-954

1. Introduction

Chronic inflammatory diseases, including type 1 diabetes, that often lead to pain are of increasing importance for health care in aging populations. Pain is a normal physiological protective mechanism to avoid tissue damage. In the periphery, it is signalled by fine C and Aδ afferent fibres that respond to noxious stimuli (mechanical, heat, cold, chemical). Chemical mediators produced during inflammation are responsible for the multiplicity of events that occur, including hyperalgesia, alterations in cell phenotype, and the expression of new molecules (neurotransmitters, enzymes, ion channels, receptors) in the peripheral nervous system and the CNS [28,12].

Kinins have been known for some time to be important mediators involved in a variety of biological effects including cardiovascular homeostasis, inflammation and nociception [5,31,32]. Kinins are amoung the first mediators released in injured tissues. They are formed from kininogens either by plasma kallikrein, which is activated early in the coagulation cascade, or tissue kallikrein, which is activated by proteases released at injured sites [4]. Their production is critical for the initiation of pain and exaggeration of sensory signalling to produce hyperalgesia and allodynia. In addition, they promote many features of inflammation including an increase in blood flow and tissue oedema as well as the release of several mediators such as prostanoids and cytokines [12,13,28]. Kinins mediate their biological effects by acting on two types of receptors, namely, B_1 and B_2. Molecular cloning of the bradykinin (BK) B_1 receptors (BKB_1-R) and the bradykinin B_2 receptors (BKB_2-R) from a variety of species including humans revealed that they belong to the family of G protein-coupled receptors [22]. The BKB_2-R, which mediates many of the physiological effects of kinins, are constitutively expressed and involved in the acute phase of the inflammation and pain response. On the other hand, the BKB_1-R, usually absent in normal

tissues, are highly induced and over-expressed during tissue injury and following treatment with inflammatory mediators like bacterial endotoxins and cytokines. They elicit persistant responses and signalling associated with slow ligand dissociation and participates in the chronic phase of the inflammation and pain response [10]. Pharmacological antagonists of the BKB_1-R-induced analgesia only in animal models of persistent inflammatory mechanical and thermal hyperalgesia [2,35,36,42,48] or of persistent visceral pain [26]. Furthermore, in a rat model of neuropathic hypersensitivity following peripheral nerve injury, treatment with BKB_1-R antagonists had an analgesic effect, 14 days after the injury [29]. Nevertheless, it has been demonstrated that the hyperalgesia induced by Freund's adjuvant was reduced in BKB_1-R-knockout mice [16].

Recent studies showed that type 1 diabetes is associated with an up-regulation of the induced BKB_1-R as a result of the over-production of cytokines, hyperglycemia and oxidative stress [43,53]. Other pharmacological evidence suggests that the BKB_1-R intervene in the pathogenesis of streptozotocin (STZ)-induced type 1 diabetes in mice, as BKB_1-R antagonists could normalize glycemia and renal function [54,55]. In addition, recent results from our laboratory showed that BKB_1-R antagonists were able to inhibit the enhanced vascular permeability associated with STZ-diabetic mice [50]. Further, we provided a clear evidence for the implication of BKB_1-R in mediating STZ-diabetic hyperalgesia in mice, since acute administration of BKB_1-R antagonists significantly and dose-dependently attenuated such hyperalgesic activity [17,18].

The goal of the present study was to evaluate the effect of chronic treatment with the selective BKB_1-R agonist desArg^{9}bradykinin (DBK) and two specific antagonists R-715 (Ac-Lys-[D-ßNal^7, Ile^8]desArg^{9}BK) [45,46] and R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ßNal^7, Ile^8]desArg^{9}BK) [34], on STZ-induced hyperalgesia in a model of type 1 diabetic mice.

2. Materials and methods

2.1. Animals

Male CD-1 mice weighing between 25-30 g (Charles River Breeding Laboratory, St. Constant, PQ, Canada) were used. The mice were housed four by cage with free access to food and water. They were maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10 %) with food and water available ad libitum. Animals were used only once in a given experiment. All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and those of the Ethics Committee of the Medical School of the University of Sherbrooke, which adhere to the guidelines of the Canadian Council on Animal Care (CCAC).

2.2. Induction of type 1 diabetes

Type 1 diabetes was induced in mice using STZ. Male CD-1 mice were given a single high i.p. dose of STZ (200 mg/kg) [33]. The induction of diabetes was confirmed by measuring the blood glucose level 96 h after STZ administration [7,27]. Blood was withdrawn from the retro-orbital sinus of mice with a 50 µl heparinized capillary tube. Blood glucose levels were determined with an automatic analyzer (Glucometer Elite XL, Bayer Incorporation, Toronto, ON, Canada) using glucose oxidase / potassium ferricyanide reagent strips. The diabetic animals used in our study had a blood glucose level higher than 20 mmol/l while the normal value ranged between 5 to 8 mmol/l [7,41].

2.3. Assessment of nociception

Hyperalgesia is an exaggerated response to noxious stimuli evoked by a hypersensitization of peripheral (C and Aδ) nociceptors in addition to a central facilitation of pain transmission at the level of the dorsal horn neurons and thalamus [44]. Pain was measured in both healthy and diabetic mice using four different thermal nociceptive tests:

2.3.1. Supra-spinal nociceptive tests

2.3.1.1. The hot plate test. The hot plate test was adapted from the technique of Eddy and Leimbach [15]. In brief, a plexiglass cylinder (20 × 14 cm) is used to confine the mouse to the anodized heated surface (275 × 263 mm) of the apparatus (IITC Hot Plate Analgesia Meter, Life Science, California, USA). The plate is adjusted to a temperature of 55 ± 0.5°C. When the pain threshold is reached the animal starts to react by licking its hind paw or to jump, and the reaction time is recorded with a built-in timer, with a maximum cut-off time of 30 sec to avoid tissue damage. Mice with latency value between 10-15 sec were selected.

2.3.1.2. The plantar stimulation test. The method of Hargreaves et al., [21] was used to assess the hind-paw nociceptive withdrawal thresholds of a free moving mouse to thermal stimuli. Mice are placed individually and trained in a plexiglass enclosures on top of a non-heated glass panel (Model IITC 336 Paw/Tail Stimulator Analgesia Meter, Life Science, California, USA) and left to acclimatize for 10 min daily for two days before starting the experiments. Light from a halogen bulb lamp (150 W) is delivered to the plantar surface of one of the mouse hind-paws through the base of the glass panel and is focused using an aluminized parabolic mirror mounted on the light source. The time taken for the mouse to lift

or lick its hind paw is noted. The intensity of the radiant heat is selected in order to reach a basal latency of 8-10 sec, and to reduce the variability. A cut-off time of 30 sec is used to avoid excessive pain. Each latency value is determined from two applications of the radiant heat stimulus, separated by 1-2 min intervals, and the mean of the two measures is taken. This test is similar to the hot plate test, except that repeated testing does not result in sensitization and the automatic endpoint minimizes the experimenter influence.

2.3.2. Spinal nociceptive tests

2.3.2.1. The tail immersion test. The tail immersion test was performed according to Coderre and Rollman [9]. The mouse is gently wrapped in a towel, held at a 45° angle to a thermostatically controlled water bath set at 52 ± 1°C. The latency between submersion of the tail and its removal from the water by the animal is recorded, with a maximum cut-off time of 10 sec to minimize tail skin tissue damage. Mice with latency value between 2.5-4.0 sec were selected.

2.3.2.1. The tail flick test. The tail flick test of D'amour and Smith [11] modified for mice was used. The mice are habituated in a plexiglass cylindrical mouse restrainer (4 cm in diameter and 8 cm long), 15 min daily for one week before starting the experiments. To measure the latency of the tail flick response, mice are gently placed in the restrainer and the tail put in the tail groove of the apparatus (Model IITC 336 Paw/Tail Stimulator Analgesia Meter, Life Science, California, USA). The tail-flick response is elicited by applying radiant heat from a halogen bulb lamp (150 W) to the dorsal surface of the animal tail. The radiant light is focused on a blackened spot in the mid region of the animal's tail (2-3 cm from the tip of the tail) and the latency between the application of the stimulation light and the flicking

of the animal's tail is recorded. When the animal flicks its tail, its exposes a photocell in the apparatus immediately below the tail and the instrument is automatically stopped and the time is recorded. A cut off time of 10 sec is used to prevent blistering. The intensity of radiation is set to provide a pre-drug tail-flick response of 4-5 sec. This test is similar to the tail immersion test with the advantage that repeated testing does not result in sensitization and the automatic detection of the endpoint minimizes the experimenter influence.

2.4. Experimental protocol

In the four thermal tests, pre-treatment latencies were determined 3 times with an interval of 24 h starting 3 days before the injection of STZ or saline and the mean was calculated in order to obtain stable pre-drug response latency. Chronic treatment with the selective BKB_1-R agonist DBK and/or its specific antagonists R-715 (400 μg/kg) and R-954 (200 μg/kg) started 4 days after STZ injection. Drugs were administered i.p. twice daily, for 7 days. By the end of this chronic treatment, the effect of BKB_1-R-related peptides on nociception in STZ-diabetic mice was evaluated using the four thermal tests, 5 h following the last dose of the agonist and/or the antagonists. Mice were divided into the following groups: *(i)* control group, treated with saline; *(ii)* group treated with STZ (200 mg/kg, i.p., once); (iii) group treated with STZ + R-715 (400 μg/kg); *(iv)* group treated with STZ + R-954 (200 μg/kg); *(v)* group treated with STZ + DBK (400 μg/kg); *(vi)* group treated with STZ + DBK + R-715; *(vii)* group treated with STZ + DBK + R-954; and *(viii, ix and x)* groups received only R-715 or R-954 or DBK, respectively. Each group was made of 6-10 mice. The selected doses for DBK, R-715 and R-954 were found to produce maximum effect as previously published in our acute studies [17,18,50].

The effect of the selected drugs on nociception was determined by converting the hot plate, the plantar stimulation, the tail immersion or the tail flick responses from latencies into MPE (Maximum Percent Effect) according to the following equation [3]:

$$(\%\ \text{MPE}) = \frac{(\text{Post - treatment latency} - \text{Pre - treatment latency})}{(\text{Cut - off time} - \text{Pre - treatment latency})} \times 100$$

2.5. Drugs

STZ was purchased from Pharmacia & Upjohn Inc. (Mississauga, ON, Canada). The BKB_1-R-related peptides: DBK (desArg^9-BK), R-715 (Ac-Lys-[D-ßNal^7, Ile^8]desArg^{9}BK) and R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ßNal^7, Ile^8]desArg^{9}BK were synthesized by Dr. Witold Neugebauer in the Institute of Pharmacology of Sherbrooke, School of Medicine, University of Sherbrooke, Canada. They were dissolved in saline and administered to mice i.p. Standard abbreviations for amino acids and peptides follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [24] and the IUPAC-IUB Joint Commission conventions on Biochemical Nomenclature [25]. Additional abbreviations are: Oic, 2S,3aS,7aS-Octahydro-1H-indole-2-carboxylic acid; α-MePhe, α-methylphenylalanine; D-ßNal, ß-(2-Naphthyl)-D-Alanine.

2.6. Statistical Analysis

Data are expressed as Mean (% MPE) ± S.E.M. and analysis of variance (ANOVA) followed by the "Student-Newman-Keuls Multiple Comparisons Test" were performed using using the Instat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). *P*-values less than 0.05 were considered significant.

3. Results

3.1. STZ-diabetes-mediated hyperalgesia

The STZ-induced type 1 diabetes was associated with a significant hyperalgesic activity as measured 11 days after STZ injection. In the hot plate and plantar stimulation tests (supra-spinal nociceptive tests), the MPE in STZ-diabetic mice was -14.77 ± 1.14 and -10.36 ± 0.22 %, respectively compared to 0.05 ± 0.01 and 0.56 ± 0.11 % in control mice (*P < 0.001*; Fig. 1-A, 1-B). Likewise, in the tail immersion and tail flick tests (spinal nociceptive tests), the MPE was -9.23 ± 0.29 and -23.78 ± 0.54 %, respectively in STZ-mice versus 0.28 ± 011 and 0.89 ± 0.20 %, in control non-diabetic mice (*P < 0.001*; Fig. 1-C, 1-D).

3.2. Effect of chronic treatment with R-715 and R-954 on STZ-diabetic hyperalgesia

Results demonstrated in Fig. 1-A, 1-B, 1-C, and 1-D, show that the chronic i.p. administration (twice daily for 7 days, starting 4 days after STZ injection) of the selective BKB_1-R antagonists R-715 (400 μg/kg) and R-954 (200 μg/kg), significantly attenuated the hyperalgesic activity developed in STZ-diabetic mice at the level of the four pain tests. R-715 produced an inhibition of 81, 61, 79 and 45% of the hyperalgesia observed in STZ-mice in the hot plate, plantar stimulation, tail immersion and tail flick tests, respectively ($P < 0.001$). The mean MPE values for the STZ group treated with R-715 versus those for the STZ controls given saline were as follows: hot plate, -2.60 ± 0.46 versus -14.77 ± 1.14 %; plantar stimulation, -4.01 ± 0.39 versus -10.36 ± 0.22 %; tail immersion, -1.95 ± 0.34 versus 9.23 ± 0.29 % and tail flick, -12.95 ± 0.56 versus -23.78 ± 0.54 %. Similarly, the more potent and stable analogue R-954, at half the dose of R-715, resulted in a similar profile of inhibition expressed as 76, 61, 66, and 47% in the four tests, respectively ($P < 0.001$).

The mean values of MPE were: hot plate, -3.63 ± 0.44 versus -14.77 ± 1.14 %; plantar stimulation, -4.01 ± 0.23 versus -10.36 ± 0.22 %; tail immersion, -3.64 ± 0.41 versus -9.23 ± 0.29 % and tail flick, -12.51 ± 0.74 versus -23.78 ± 0.54 % for the STZ/R-954 group compared to the STZ group. It is noteworthy that neither R-715 nor R-954 administration to control mice had significant effect on the reaction times in each of the four tests (data not shown).

Moreover, the co-administration of either R-715 or R-954 with the BKB_1-R agonist DBK resulted in a complete reversal of the potentiating effect induced by DBK on STZ-diabetic hyperalgesia. Chronic administration of DBK (400 μg/kg; i.p. twice daily for the same period of time) potentiated by 35, 60, 68, and 57% the hyperalgesic effect observed in STZ-mice ($P < 0.001$; Fig. 1-A, 1-B, 1-C, and 1-D). The mean values of the reaction time, expressed as MPE, were: -19.89 ± 1.18 versus -14.77 ± 1.14 %; -16.66 ± 0.49 versus -10.36 ± 0.22 %; -15.56 ± 0.56 versus -9.23 ± 0.29 % and -37.49 ± 1.38 versus -23.78 ± 0.54 % for the STZ/DBK group versus the STZ group in the hot plate, plantar stimulation, tail immersion and tail flick tests, respectively. However, injection of DBK together with R-715 or R-954 to STZ-diabetic mice, showed a significant increase in the reaction time of the four tests compared to that in STZ-mice receiving DBK alone. The mean MPE values obtained in the STZ/DBK/R-715 group were as follows: hot plate, -3.18 ± 0.48 %; plantar stimulation, -4.22 ± 0.24; tail immersion, -5.24 ± 0.31 and tail flick –17.46 ± 1.53. On the other hand, combined treatment of STZ-mice with DBK and R-954 gave the following MPE values: -5.51 ± 0.60, -4.22 ± 0.36, -5.85 ± 0.34 and -15.62 ± 0.67 in the four tests, respectively compared to -19.89 ± 1.18, -16.66 ± 0.49, -15.56 ± 0.56 and -37.49 ± 1.38 in the STZ/DBK group ($P < 0.001$).

4. Discussion

In the present study, we demonstrated that chronic administration of the two selective BKB_1-R antagonists, R-715 [45,46] and R-954 [34], significantly attenuated type 1 diabetic hyperalgesia in STZ-mice as evaluated by two types of thermal noxious tests, the hot plate and the plantar stimulation tests (supra-spinal nociceptive reflexes) as well as the tail immersion and the tail flick tests (spinal nociceptive reflexes). In addition, the same antagonists completely reversed the potentiating effect of the BKB_1-R agonist, DBK on STZ-induced hyperalgesia.

Current evidence indicates that type 1 diabetes is due to an autoimmune process that is directed against the beta cells of the pancreas and develops in genetically predisposed individuals. It is associated with the over-production of cytokines, including interleukin-1β and tumour necrosis factor-α (TNF-α), that leads to the destruction of pancreatic islet β-cells [43]. These cytokines are believed to be implicated in the induction of the BKB_1-R [32,38], through activation of the mitogen-activated protein kinase (MAP kinase) and the nuclear transcriptional factor kappa B (NF-κB) [6]. In addition, hyperglycemia and the resulting oxidative stress can also activate the NF-κB [53], leading to BKB_1-R expression [32]. Pharmacological evidence further suggests that the BKB_1-R intervene in the pathogenesis of STZ-induced type 1 diabetes in mice and the development of its complications. To that effect, Zuccollo et al., [54] demonstrated that BKB_1-R antagonists normalize glycemia and renal function. When administered with STZ, they reversed the elevation of blood glucose level and prevented the renal abnormalities, including increased urine volume and increased excretion of protein, nitrite and kallikrein. Also, it has been established that the BKB_1-R are over-expressed in the stomach of diabetic mice since the sensitivity of the stomach fundus to DBK was substantially increased in these animals [40]. Furthermore, it has been reported

that the BKB_1-R play a determinant role in the increased vascular permeability associated with type 1 diabetes as BKB_1-R antagonists could inhibit the enhanced permeability as measured by extravasation of Evans Blue dye in several mouse tissues (liver, pancreas, duodenum, ileum, kidney) [50]. Moreover, the BKB_1-R were reported to be induced in kidneys and spinal cord of rats treated three weeks earlier with STZ [8,30].

However, no other groups presented direct evidence for the involvement of the BKB_1-R in the hyperalgesic activity induced in diabetic animals. We recently reported that STZ-diabetic mice developed a well defined hyperalgesia that was assessed in different types of thermal nociceptive tests. We also demonstrated that acute i.p. administration of specific BKB_1-R antagonists R-715 and R-954, inhibited such hyperalgesic activity in a dose-dependent manner, in addition to reversing the DBK potentiating effect on hyperalgesia [17,18]. The findings in the present chronic study provide further support for the implcation of the BKB_1-R in dibebetes-mediated hyperalgesia. Therefore, exaggeration of sensory signaling and pain sensation in STZ-diabetic mice could be explained by the BKB_1-R-mediated pathway. First, it is known that BKB_1-R, selectively activated by BKB_1-R agonists, are normally absent or of little activity under normal physiological conditions [10], whereas, such selective agonists are effective upon over-expression of the BKB_1-R in pathological conditions as alongside diabetes. Secondly, in inflammatory conditions, such as type 1 diabetes, chronic activation of the inducible BKB_1-R is likely to be amplified by the accumulation of the DBK, the metabolite resulting from the degradation of BK, at the site of inflammation [32]. This can be attributed in part to the up-regulation of carboxypeptidase M (kininase I, the enzyme responsible for the metabolism of BK to DBK), which would increase the endogeno level of DBK [49].

Thus, DBK may directly induce hyperalgesia by stimulating the inducible BKB_1-R on sensory neurons to release substance P, calcitonin gene-related peptide (CGRP), neurokinin A and other nociceptive neurotransmitters (Petersen et al., 1998). This could be sensitized by the action of prostaglandins or other mediators released from other cells by the activation of BKB_1-R. A second mechanism could be proposed involving DBK activation of the BKB_1-R induced on cells other than the sensory neurons (macrophages, fibroblasts or endothelial cells) with the subsequent release of mediators (prostaglandins, cytokines and nitric oxide) that sensitize the nociceptors [14]. A third hypothesis suggests the stimulation of the sympathetic nerves by kinins to release prostanoids, sympathetic transmitters or other mediators that sensitize nociceptive nerve terminals, leading to hyperalgesia. Finally, the increase in vascular permeability induced by the BKB_1-R might be implicated in the hyperalgesic effect through the extravasation of blood constituents that might have a sensitizing effect on pain perception.

The BKB_1-R antagonists used in our studies, R-715 and its more potent and stable analogue R-954, are believed to attenuate the hyperalgesic activity observed in diabetic mice by competitively inhibiting the inducible BKB_1-R. The classical BKB_1-R antagonists such as desArg^{10}Hoe 140 [52], [Leu^8]desArg^{9}BK and Lys-[Leu^8]desArg^{9}BK [1], were found to act as partial agonists in various segments of the intestine and the urinary bladder of the mouse [1] and of the rat colon epithelium [51]. A modification of the structure of these classical BKB_1-R antagonists has led to new compounds including R-715 (pA_2 = 7.0 –8.4 in murine stomach, rabbit aortas and human umbilical veins) [1,19,34]. In R-715, the substitution with Ac-Lys in N-terminal and D-βNal in position 7, as well as Ile in position 8 led to one of the most active and selective BKB_1-R antagonist with high affinity and moderate enzymatic resistance [19]. Additional chemical modifications from the lead compound R-715 gave R-954 (pA_2 = 8.4-8.6 in rabbit aortas and human umbilical veins) [34], which proved to be more

potent and more resistant to enzymatic degradation as a result of the α-methylation of Phe in position 5 and the substitution with Oic in position 2 and the addition of Ac-Orn instead of Ac-Lys at the N-terminal.

Taken together, the potentiation of diabetic hyperalgesia produced by the chronically administered BKB_1-R agonist DBK, once this receptor is expressed *de novo* under pathological conditions including type 1 diabetes, could be explicated by persistent signalling due to lack of ligand dissociation, desensitization and receptor internalization. Moreover, long-term stimulation of this receptor actually leads to increased expression. On the other hand, since antagonist-stimulated intracellular sequestration has been recently highlighted as a possible mechanism for antagonist-induced desensitization of the G protein-coupled receptors including the cholecystokinin receptor [47], the BKB_2-R [23], the 5-hydroxytryptamine (2A) receptor [20] and the neuropeptide Y Y(1) receptor [39], it could be thus proposed that the chronic effect observed with the BKB_1-R antagonists, R-715 and R-954 in attenuating diabetes-induced hyperalgesia might be due to long-lasting disappearance of cell surface receptors through a pathway distinct from the classical endocytic/recycling pathway followed upon agonist stimulation.

In conclusion, the results provided in this chronic study further confirm a pivotal role for the BKB_1-R in the development of inflammatory hyperalgesia associated with type 1 diabetes and support the potential clinical therapeutic utility of BKB_1-R antagonists in the management of type 1 diabetic complications.

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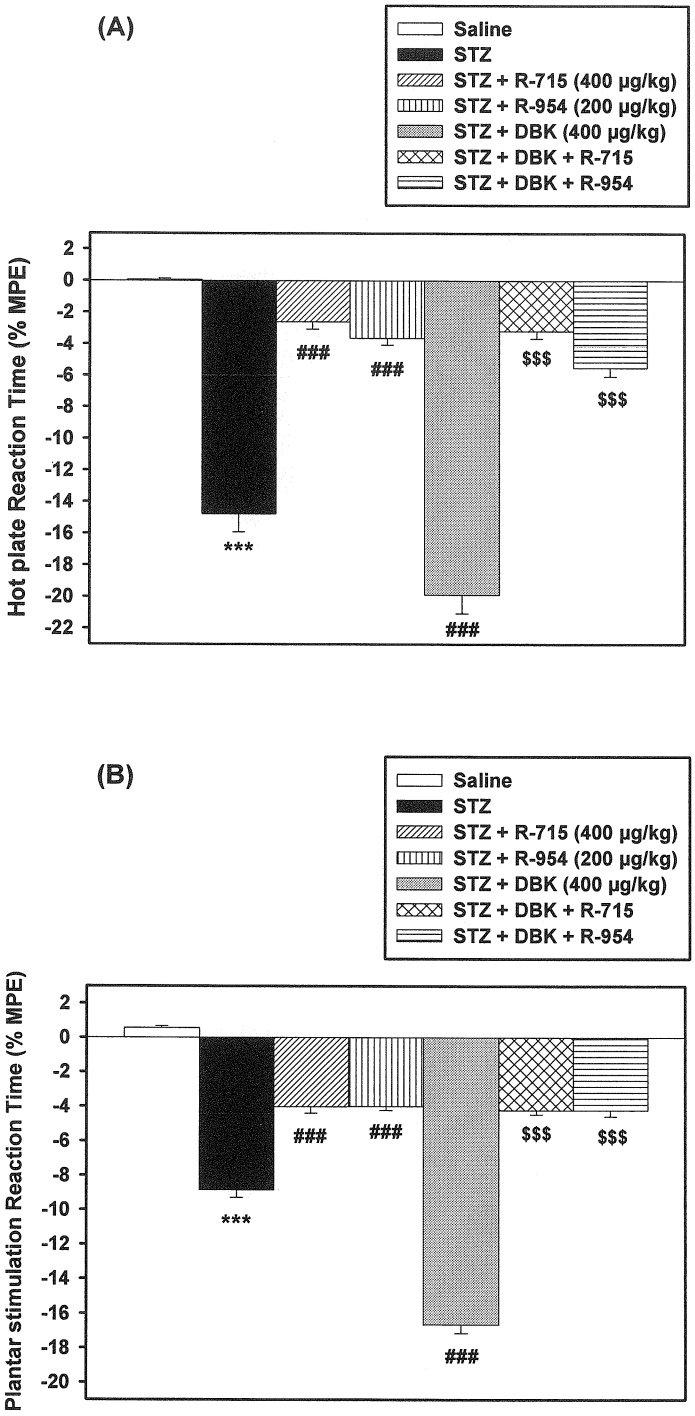
Legend for figure

Fig. 1. Effect of chronic administration of R-715 or R-954, and their administration with DBK on nociception in streptozotocin (STZ)-diabetic mice in the hot plate (A), the plantar stimulation (B), the tail immersion (C) and the tail flick (D) tests. Type 1 diabetes was induced in CD-1 mice using streptozotocin (STZ; 200 mg/kg, i.p.). The specific BKB_1-R antagonists, R-715 (400 μg/kg) and R-954 (200 μg/kg) as well as the selective BKB_1-R agonist DBK (400 μg/kg) were administered i.p. twice daily for 7 days starting from day 4 after STZ injection. On day 11 following the induction of the disease, the four thermal nociceptive tests were carried out. Data are expressed as mean % MPE ± S.E.M. (n = 6-10). MPE = Maximum Percent Effect. n = number of animals. *** significantly different from the saline group at $P < 0.001$; ### significantly different from the STZ group at $P < 0.001$, $ significantly different from the STZ/DBK group $P < 0.001$



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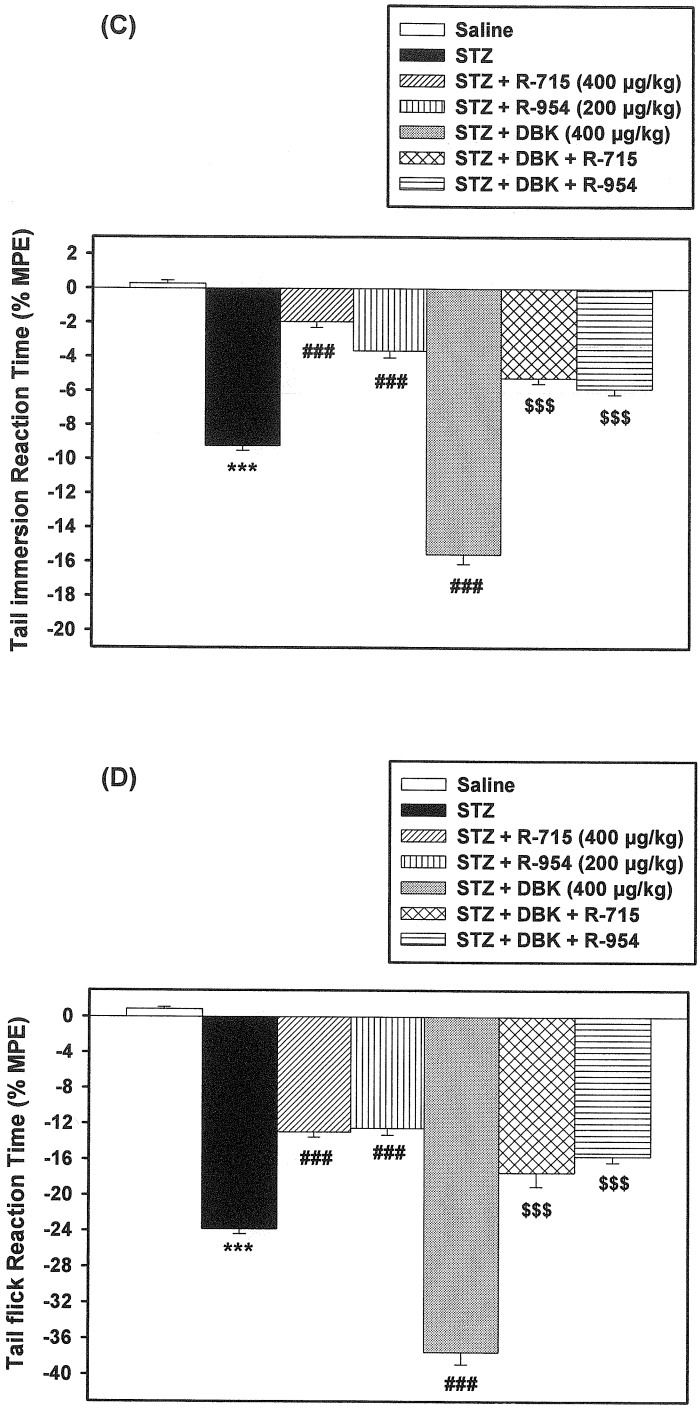

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RESULTS

Article 4

Pathways for the bradykinin B_1 receptor-mediated diabetic hyperalgesia in mice

B. H. Gabra and P. Sirois

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Abstract. *Objective:* Experimental evidence has shown that the bradykinin B_1 receptor (BKB_1-R) is involved in the development of hyperalgesia associated with diabetes since specific BKB_1-R antagonists significantly inhibited the hyperalgesic activity observed in streptozotocin (STZ)-mice in thermal nociceptive tests.

Materials and Methods: The involvement of the nitric oxide (NO), the substance P (SP) and the calcitonin gene-related peptide (CGRP) pathways in mediating BKB_1-R-induced hyperalgesia was evaluated. Diabetes was induced in male CD-1 mice by injecting STZ (200 mg/kg; i.p.). Nociception was assessed using the hot plate and tail immersion tests, one week following the injection of STZ.

Results: The nitric oxide synthase (NOS) inhibitors (L-NNA, 20 mg/kg; L-NMMA, 30 mg/kg and AGUA, 50 mg/kg; i.p.), the SP antagonists (sendide and L-732,138, 100 μg/kg; i.v.) and the CGRP antagonist (hCGRP8-37, 100 μg/kg; i.v.) significantly attenuated the hyperalgesic activity and also reversed the potentiating effect of the BKB_1-R agonist DBK on diabetic hyperalgesia in STZ-mice.

Conclusions: These results support the involvement of BKB_1-R in the development of diabetic hyperalgesia in STZ-mice through activation of the NO, SP and CGRP pathways.

Key words: Type 1 diabetes – Hyperalgesia – Kinins - Bradykinin B_1 receptors - Nitric oxide - Substance P - Calcitonin gene-related peptide

Introduction

Kinins are key mediators of a variety of biological effects such as cardiovascular homeostasis, inflammation and nociception. The bradykinin (BK) B_1 receptor (BKB_1-R), usually absent in normal tissues, is over-expressed in many pathological conditions including type 1 diabetes, where the over-production of cytokines, the hyperglycemia and the oxidative stress are critical factors for its up-regulation (Marceau et al., 1998; Couture et al., 2001). It has been reported that the BKB_1-R plays an important role in hyperglycemia and renal abnormalities associated with type 1 diabetes (Zucollo et al., 1996; 1999).

We have recently demonstrated that the BKB_1-R is also involved in the altered vascular permeability (Simard et al., 2002) and in the development of hyperalgesia associated with steptozotocin (STZ)-diabetic mice (Gabra and Sirois, 2002; 2003). Acute administration of the selective BKB_1-R antagonist R-954 significantly inhibited the enhanced vascular permeability in most tissues of STZ-mice. Both acute and chronic administration of the specific BKB_1-R antagonists R-715 and its more potent analogue R-954 significantly inhibited the hyperalgesic activity observed in STZ-mice in selected thermal nociceptive tests. They also reversed the potentiating effect of the BKB_1-R agonist, desArg^{9}BK (DBK) on diabetic hyperalgesia (Gabra and Sirois, 2002; 2003). Since several pathways might be involved in the mediation of the BKB_1-R-induced hyperalgesia, the present study aimed to evaluate the involvement of the nitric oxide (NO) and the pro-inflammatory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP) as significant pain mediators, in transmitting BKB_1-R-induced hyperalgesia in diabetic mice.

Materials and Methods

Animals: Male CD-1 mice weighing between 25-30 g (Charles River Breeding Laboratory, St. Constant, PQ, Canada) were used. The mice were housed four by cage with free access to food and water. They were maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10 %) with food and water available *ad libitum*. Animals were used only once in a given experiment. All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines and were approved by the Animal Care Committee of University of Sherbrooke.

Induction of type 1 diabetes: The STZ model is commonly used to study the cardiovascular and neuropathic complications of type 1 diabetes. Mice were given a single i.p. dose (200 mg/kg) of STZ (McEvoy et al., 1984). Diabetes was confirmed by measuring blood glucose level 72 h after STZ. The diabetic animals used in our study had a blood glucose level higher than 20 mmol/L.

The hot plate test: A hot plate test derived from that of Eddy and Leimbach (Eddy and Leimbach, 1953) was used. Mice were placed on a IITC Hot Plate Analgesia Meter (Life Science, California, USA) adjusted at 55 ± 0.5°C and the latency of the reaction to this nociceptive stimulus (number of seconds before the animal started licking the hind paw or jumping) was quantified. Only mice with basal latency value between 10-15 sec were selected and a maximum cut-off time of 30 sec was observed to avoid excessive pain.

The tail immersion test: The tail immersion test was performed according to Coderre and Rollman (Coderre and Rollman, 1983). The mouse is gently wrapped in a towel, held at a 45° angle to a thermostatically controlled water bath set at 52 ± 1°C. The latency between submersion of the tail and its removal from the water by the animal is recorded, with a maximum cut-off time of 10 sec to avoid tail skin tissue damage. Mice with latency value between 2.5-4.0 sec were selected.

Experimental protocol: Pre-treatment latencies were determined 3 times with an interval of 24 h starting 3 days before the injection of STZ or saline in order to obtain stable pre-drug response (basal latency). Seven days following the injection of STZ, the selective BKB_1-R agonist, DBK (desArg^{9}BK; 400 μg/kg) and its specific antagonists R-715 (400 μg/kg) and R-954 (200 μg/kg) as well as the NO synthase (NOS) inhibitors (N^{ω}-nitro-L-arginine, L-NNA; N^{ω}-monomethyl-L-arginine, L-NMMA; aminoguanidine, AGUA) were given i.p., whereas the SP antagonists sendide and L-732,138 as well as the CGRP antagonist hCGRP8-37, were administered i.v. The selected doses for DBK, R-715 and R-954 were found to produce maximum effect as previously published (Gabra and Sirois, 2002; 2003; Simard et al., 2002). The effect of acute administration of these drugs on nociception was determined 20 min later. The hot plate and tail immersion responses are presented as % of basal latency.

Drugs: STZ was purchased from Pharmacia & Upjohn Inc. (Mississauga, ON, Canada). DBK, R-715 (Ac-Lys-[D-ß Nal^7, Ile^8]desArg^{9}BK) and R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ß Nal^7, Ile^8]desArg^{9}BK) were synthesized by Dr. Witold Neugebauer in the Institute of Pharmacology

of Sherbrooke, School of Medicine, University of Sherbrooke, Canada). L-NNA, L-NMMA, AGUA and hCGRP8-37 were purchased from Sigma Chem. (St. Louis MO, USA), whereas L-732,138 (Ac-Trp-3,5bis(trifluoromethyl)benzyl ester) and sendide (Tyr-D-Phe-Phe-D-His-Leu-Met-NH_2) were purchased from Bachem (Torrance, CA, USA).

Statistical analysis: Data are expressed as means % of basal latency ± S.E.M. and analysis of variance (ANOVA) followed by the "Student-Newman-Keuls multiple comparisons test" was performed using the Instat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). A probability (p) value less than 0.05 was considered significant.

Results

Induction of type 1 diabetes using STZ was associated with the development of significant hyperalgesic activity as measured 7 days following STZ. The % of basal latency was 79.54 ± 0.58 and 65.63 ± 1.16 % for the diabetic mice compared to 99.55 ± 0.04 and 98.63 ± 0.23 % for the control non-diabetic group in the hot plate (supra spinal) and the tail immersion (spinal) tests, respectively ($p < 0.001$; Fig. 1a and 1c).

Injection of the selective BKB_1-R antagonists R-715 or R-954 did not affect the nociceptive threshold in control healthy mice (data not shown). However, R-715 and its more potent and stable analogue R-954 significantly increased hot plate and tail immersion latencies observed in diabetic mice to normal values observed in the saline-treated group. R-715 (400 µg/kg, i.p.) restored the % of basal latency to 98.48 ± 0.21 and 97.87 ± 0.20 % whereas R-954 (200 µg/kg, i.p.) increased the % of basal latency to 99.31 ± 0.16 and 97.49 ± 0.30 % in the hot plate and the tail immersion tests, respectively ($p < 0.001$; Fig. 1a and 1c).

The NOS inhibitors (L-NNA, 20 mg/kg; L-NMMA, 30 mg/kg and AGUA, 50 mg/kg; i.p.), the SP antagonists (sendide and L-732, 138, 100 µg/kg; i.v; potent and selective antagonists to SP at neurokinin NK-1 receptors) and the CGRP antagonist (hCGRP8-37, 100 µg/kg; i.v.) were administered in order to investigate the possible implication of the NO, SP and the CGRP pathways in the mediation of BKB_1-R-induced hyperalgesia. They significantly attenuated diabetic hyperalgesia in both thermal nociceptive tests. The NOS inhibitors increased the % of basal latency to almost 90 and 85 % in the hot plate and tail immersion tests, respectively ($p < 0.001$; Fig. 1a and 1c). On the other hand, the SP antagonists restored the % of basal latency values to 92 and 89 % while the CGRP antagonist to 95 and 87 % in the two types of pain tests, respectively ($p < 0.001$; Fig. 1a and 1c). It is noteworthy that the NOS

inhibitors, the SP antagonists and the CGRP antagonist administered to control non-diabetic mice had no significant effect on the reaction time in both pain tests (data not shown). In addition, the selected inhibitors and/or antagonists reversed the potentiating effect of the BKB_1-R agonist, DBK (400 μg/kg; i.p.) on diabetic hyperalgesia. DBK decreased the % of basal latency to 60 and 52 % in the hot plate and tail immersion tests, respectively ($p < 0.001$; Fig. 1b and 1d). The % of basal latency was increased to almost 90 and 85 % in the L-NNA, L-NMMA, AGUA, sendide, L-732-138 and hCGRP8-37 groups compared to the DBK-treated group in the hot plate and tail immersion tests, respectively ($p < 0.001$; Fig. 1b and 1d). It is important to mention that DBK increased the hyperalgesic activity only in STZ mice and not in control non-diabetic mice.

Discussion

The hyperalgesic effect observed in diabetic mice and its potentiation by the BKB_1-R agonist DBK, as well as the antinociceptive effect of the BKB_1-R antagonists, R-715 and R-954, could be explained by the BKB_1-R-mediated pathway (Gabra and Sirois, 2002; 2003). The BKB_1-R, selectively activated by BKB_1-R agonists, is normally absent or of little activity under normal physiological conditions (Couture et al., 2001), whereas, such selective agonists are effective upon over-expression of the BKB_1-R in pathological conditions as alongside diabetes. Chronic activation of the inducible BKB_1-R in type 1 diabetes is likely to be amplified by the accumulation of the DBK, the metabolite resulting from the degradation of BK, at the site of inflammation. Thus, DBK may directly induce hyperalgesia by stimulating the inducible BKB_1-R on sensory neurons to release SP, CGRP, neurokinin A and other nociceptive neurotransmitters (Couture et al., 2001). This could be sensitized by the action of prostaglandins or other mediators released from other cells by the activation of BKB_1-R. A second mechanism could be proposed involving DBK activation of the BKB_1-R induced on cells other than the sensory neurons (macrophages, fibroblasts or endothelial cells) with the subsequent release of mediators (prostaglandins, cytokines and nitric oxide) that sensitize the nociceptors (Dray and Perkins, 1997). It could be also suggested that stimulation of sympathetic nerves as a result of DBK binding to the inducible BKB_1-R will cause the release of neuropeptides, prostanoids, sympathetic transmitters or other mediators that sensitize nociceptive nerve terminals, leading to hyperalgesia (Walker et al., 1995).

In the present study we demonstrated that inhibitors and/or antagonists of the NOS and the pro-inflammatory neuropeptides, SP and CGRP were able to attenuate the hyperalgesic activity induced by BKB_1-R in diabetic mice. These results provide further support for the involvement of the BKB_1-R in the development of diabetic hyperalgesia in STZ-mice through the subsequent activation of the NO, SP and CGRP pathways.

Acknowledgements

The authors would like to acknowledge Dr. Domenico Regoli and Dr. Witold Neugebauer for the supply and synthesis of DBK, R-715 and R-954.

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Fig. 1. Effect of acute administration of the BKB_1-R antagonists, NOS inhibitors, SP antagonists and CGRP antagonist **(a, c)** and their combination with the selective BKB_1-R agonist, DBK **(b, d)** on the hyperalgesic activity in type 1 diabetic mice measured by the hot plate **(a, b)** and the tail immersion **(c, d)** tests. Diabetes was induced in CD-1 mice using STZ (200 mg/kg, i.p.). On day 7 following STZ injection, R-715 (400 μg/kg), R-954 (200 μg/kg), DBK (400 μg/kg), L-NNA (20 mg/kg), L-NMMA (30 mg/kg), AGUA (50 mg/kg) were injected i.p. whereas sendide (100 μg/kg), L-732,138 (100 μg/kg) and hCGRP8-37 (100 μg/kg) were administered i.v. to diabetic mice. The hot plate and the tail immersion latencies at 20 min post-drug for each animal are expressed as mean % of basal latency ± S.E.M. (n=6-10). Values significantly different from saline at * $p < 0.001$, from STZ at $^{\#}p < 0.001$ and from STZ/DBK at $^{+}p < 0.001$.

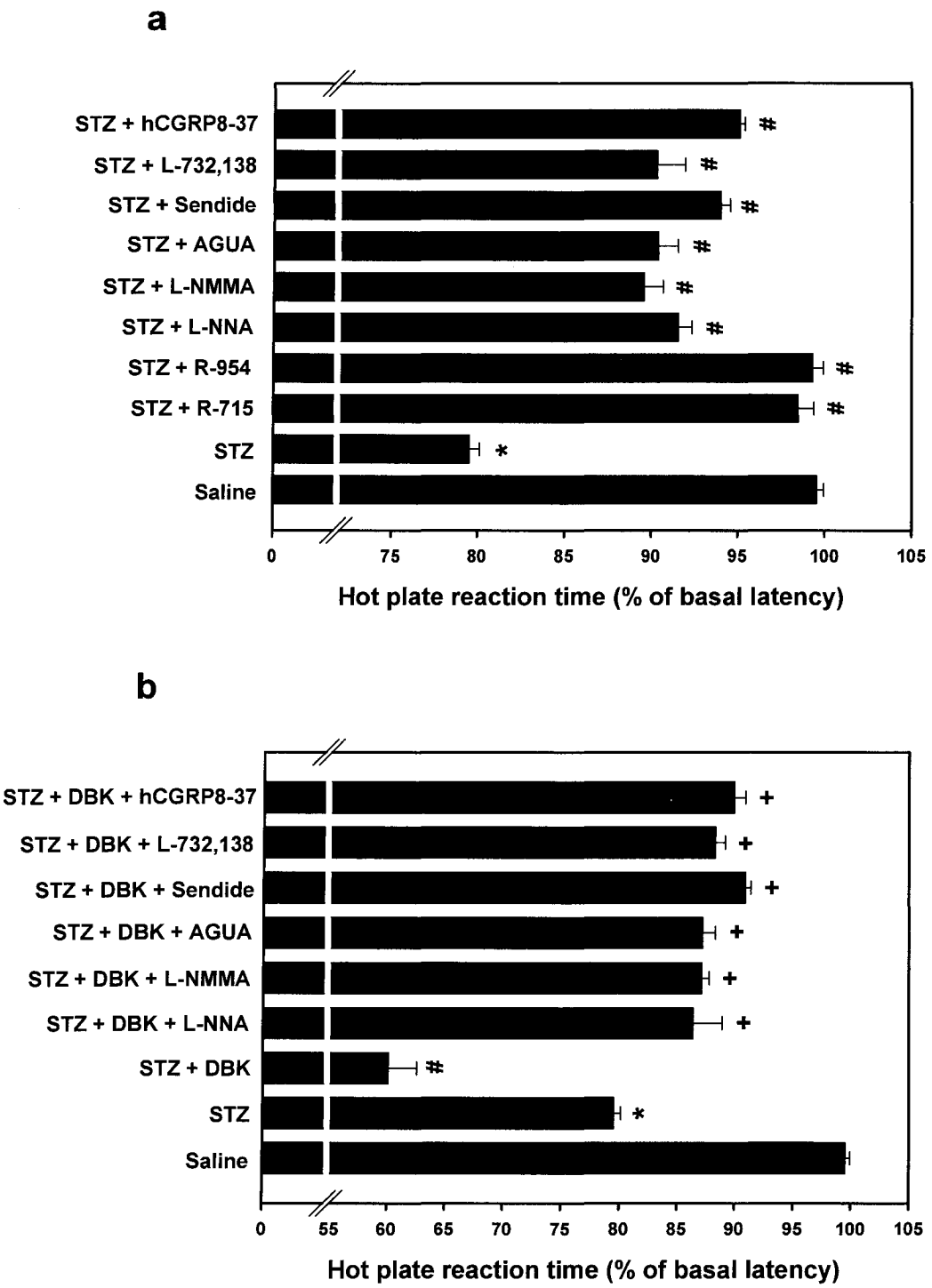

Fig. 1. Effect of acute administration of the BKB_1-R antagonists, NOS inhibitors, SP antagonists and CGRP antagonist (a, c) and their combination with the selective BKB_1-R agonist, DBK (b, d) on the hyperalgesic activity in type 1 diabetic mice measured by the hot plate (a, b) and the tail immersion (c, d) tests.

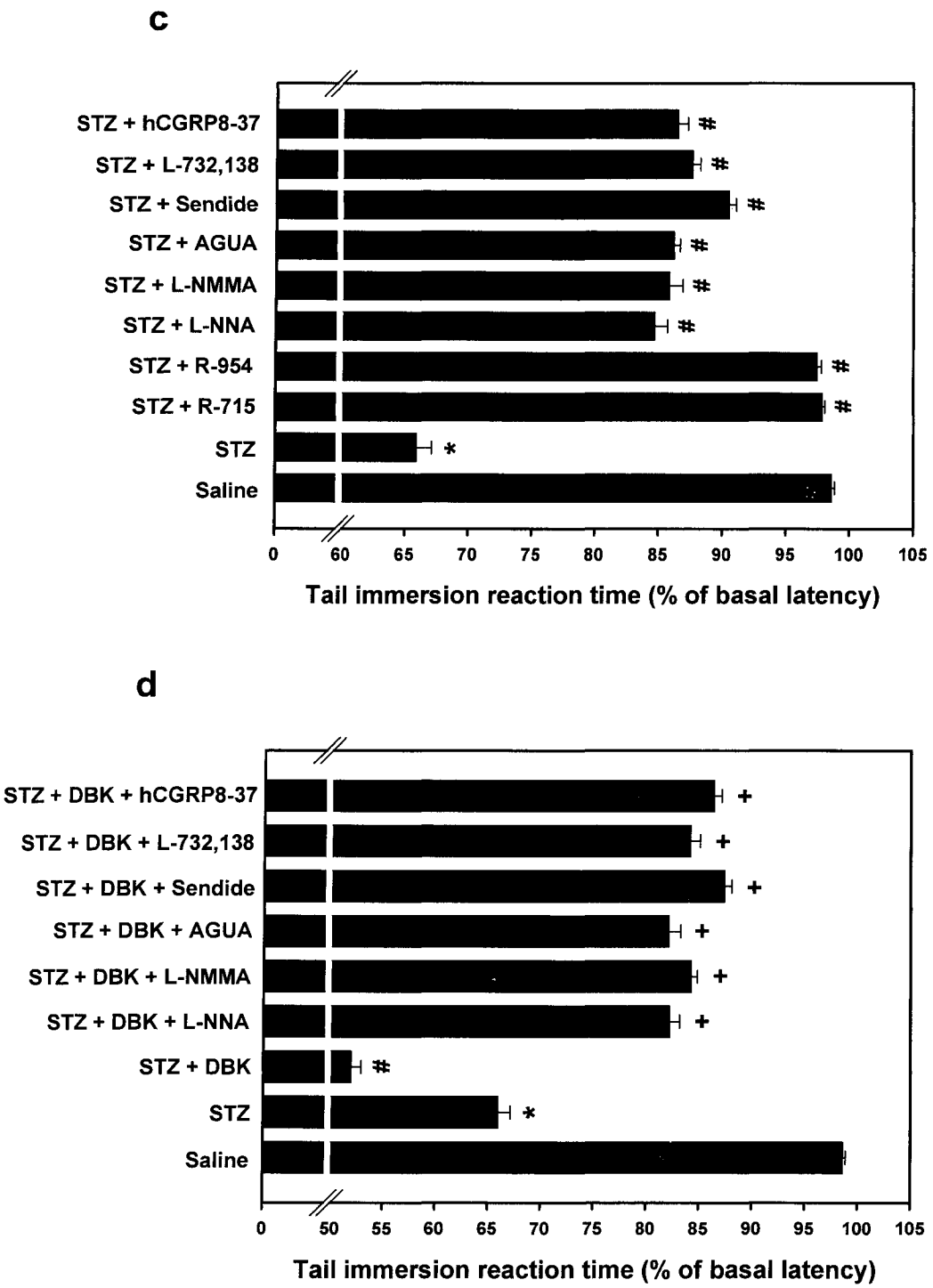

Fig. 1. Effect of acute administration of the BKB_1-R antagonists, NOS inhibitors, SP antagonists and CGRP antagonist (a, c) and their combination with the selective BKB_1-R agonist, DBK (b, d) on the hyperalgesic activity in type 1 diabetic mice measured by the hot plate (a, b) and the tail immersion (c, d) tests.

RESULTS

Article 5

Absence of diabetic hyperalgesia in bradykinin B_1 receptor-knockout mice

Bichoy H. Gabra, Vanessa F. Merino, Michael Bader, João B. Pesquero, Pierre Sirois

Regulatory Peptides 127 (2005) 245-248

Abstract

Experimental evidence has shown that the inducible bradykinin (BK) B_1 receptor (BKB_1-R) subtype is involved in the development of hyperalgesia associated with type 1 diabetes. Selective BKB_1-R antagonists inhibited, whereas selective agonists increased the hyperalgesic activity in diabetic mice in thermal nociceptive tests. Here we evaluate the development of diabetic hyperalgesia in a BKB_1-R-knockout (KO (-/-)) genotype compared to wild-type (WT (+/+)) mice. The BKB_1-R-KO (-/-) mice were backcrossed for 10 generations to C57BL/6 genetic background before use in the experiments. Diabetes was induced by streptozotocin (STZ) and thermal nociception was assessed by the hot plate and tail immersion tests. The hyperalgesia observed in wild type mice was totally absent in the BKB_1-R-KO (-/-) mice. Furthermore, the selective BKB_1-R agonist, desArg^{9}BK, significantly increased the hyperalgesic activity in diabetic WT (+/+) mice but had no effect on nociceptive responses in diabetic BKB_1-R-KO mice. Taken together, the results confirm the crucial role of the BKB_1-R, up-regulated alongside inflammatory diabetes, in the development of diabetes-induced hyperalgesia.

Keywords: type 1 diabetes; hyperalgesia; kinins; bradykinin B_1 receptor; desArg^{9}bradykinin; R-715; R-954.

1. Introduction

Kinins are key mediators implicated in a variety of biological effects such as cardiovascular homeostasis, inflammation and nociception. The bradykinin (BK) B_1 receptor (BKB_1-R) subtype, generally silent or absent in healthy states, is induced or activated by pathological conditions including type 1 diabetes, where the over-production of cytokines, the hyperglycemia and the oxidative stress are critical factors for its up-regulation [1].

The BKB_1-R was reported to play a crucial role in hyperglycemia and renal abnormalities [2] as well as in altered vascular permeability [3] associated with type 1 diabetes. We have recently demonstrated that the BKB_1-R is also involved in the development of hyperalgesia measured in steptozotocin (STZ)-induced type 1 diabetes of murine models [4, 5]. Both acute and chronic administration of the selective BKB_1-R antagonists R-715 and its more potent and stable analogue R-954 significantly inhibited the hyperalgesic activity observed in STZ-mice in selected thermal nociceptive tests. They also reversed the potentiating effect of the selective BKB_1-R agonist, desArg^{9}BK (DBK), on diabetic hyperalgesia [4, 5]. The present study aimed at establishing the potential pathophysiological role of the BKB_1-R in diabetic hyperalgesia. Nociceptive responses to the hot plate (supra spinal) and the tail immersion (spinal) tests were evaluated in BKB_1-R-knockout (KO (-/-)) versus wild-type (WT (+/+)) mice prior to and 1 week following the induction of type 1 diabetes with STZ.

2. Materials and methods

2.1. Disruption of the BKB_1-R Gene

The BKB_1-R-KO (-/-) mice were generated as described in [6]. They were backcrossed for 10 generations to C57BL/6 genetic background before use in these experiments. C57BL/6 mice were used as WT (+/+).

2.2. Induction of type 1 diabetes

Type 1 diabetes was induced in male C57BL/6 mice (25-35 g; Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany). Both KO (-/-) and WT (+/+) mice were given a single i.p. dose (200 mg/kg) of STZ [7]. Diabetes was confirmed by measuring blood glucose level, 72 h after STZ, with an automatic analyser (Glucometer Elite XL, Bayer Incorporation, Toronto, Ontario, Canada) using glucose oxidase / potassium ferricyanide reagents strips. Only diabetic mice with a blood glucose level higher than 20 mmol/L were used in the experiments. The normal blood glucose level in non-diabetic controls ranged from 5 to 8 mmol/L.

2.3. The hot plate test

A hot plate test derived from that of Eddy and Leimbach [8] was used. Mice were placed on a IITC Hot Plate Analgesia Meter (Life Science, California, USA) adjusted at 55 ± 0.5°C. The hot plate response was the latency observed from the time the mouse was placed on the heated surface until the first overt behavioural sign of nociception such as (i) the mouse licking a hind paw, (ii) vocalization, or (iii) an escape response. The timer was stopped

by a foot-operated pedal and the mouse immediately removed from the hot plate. A maximum cut-off time of 30 sec was observed to avoid excessive pain.

2.4. The tail immersion test

The tail immersion test was performed according to Coderre and Rollman [9]. The mouse was gently wrapped in a towel, held at a 45° angle to a thermostatically controlled water bath set at 52 ± 1°C. The latency between submersion of the tail and its removal from the water by the animal was recorded, with a maximum cut-off time of 10 sec.

2.5. Experimental protocol

Pre-treatment latencies were determined in both KO (-/-) and WT (+/+) mice, 3 times with an interval of 24 h starting 3 days before the injection of STZ or saline, in order to obtain a stable pre-drug response (basal latency). Seven days following the injection of STZ, the selective BKB_1-R agonist, DBK (400 μg/kg) and its selective antagonists R-715 (400 μg/kg) or R-954 (200 μg/kg) were administered i.p. to KO (-/-) and WT (+/+) mice and nociception was tested 20 min later. The hot plate and tail immersion responses are presented as % of basal latency. The selected doses for DBK, R-715 and R-954 were found to produce maximum effect as previously reported [4, 5].

2.6. Drugs

STZ was purchased from Pharmacia & Upjohn Inc. (Mississauga, ON, Canada). DBK ($desArg^9BK$), R-715 (Ac-Lys-[D-ß Nal^7, Ile^8]$desArg^9BK$) [10] and R-954

(Ac-Orn-[Oic^2, α-MePhe^5, D-ß Nal^7, Ile^8]desArg^{9}BK) [11] were supplied by IPS Pharma Inc. (Sherbrooke, PQ, Canada).

2.7. Statistical analysis

Data are expressed as means % of basal latency ± S.E.M. and analysis of variance (ANOVA) followed by the "Student-Newman-Keuls multiple comparisons test" were performed using the Instat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). A probability *P* value less than 0.05 was considered significant.

3. Results

Basal hot plate and tail immersion latencies were not different between the two genotypes (BKB_1-R-KO (-/-) versus WT (+/+) mice) (Fig. 1a and 1b). Induction of type 1 diabetes with STZ was associated with significant hyperalgesic activity (increase in nocifensive behaviour) in the WT (+/+) compared to the BKB_1-R-KO (-/-) mice in both nociceptive tests. The percent of hot plate basal latency, measured 7 days following STZ injection, was 71.05 ± 1.16 % for the diabetic WT (+/+) mice compared to 99.26 ± 0.10 % for the non-diabetic WT (+/+) mice ($P < 0.001$; Fig. 1a). On the other hand, we demonstrate for the first time that the diabetic mice lacking the BKB_1-R did not show significant difference in their hot plate latencies compared to non-diabetic controls ($P > 0.05$; Fig. 1a). Similarly, the percent of tail immersion basal latency was significanty decreased in diabetic (76.11 ± 0.84 %) compared to non-diabetic WT (+/+) mice (98.62 ± 0.29 %) ($P < 0.001$; Fig. 1b), whereas the tail immersion responses from the diabetic BKB_1-R-KO (-/-) were not different from those from non-diabetic controls ($P > 0.05$; Fig. 1b).

The exogenous administration of the selective BKB_1-R agonist, DBK (400 μg/kg; i.p.) markedly increased the hyperalgesic activity observed in WT (+/+) diabetic mice but had no significant effect on all nociceptive responses in diabetic BKB_1-R-KO (-/-) mice (Fig. 1a and 1b).

Acute administration of the selective BKB_1-R antagonists R-715 or R-954 at the doses of 400 and 200 μg/kg i.p., respectively, to the diabetic C57BL/6 WT (+/+) mice abolished hyperalgesia and restored the hot plate and tail immersion latencies to their normal values, whereas the same antagonists had not effect on the basal latencies of the two tests

in the diabetic BKB_1-R-KO (-/-) mice (Fig. 1a and 1b). It is noteworthy that DBK, R-715 and R-954 tested in both C57BL/6 non-diabetic WT (+/+) and BKB_1-R-KO (-/-) mice did not induce significant changes in the hot plate and tail immersion reaction times (data not shown).

4. Discussion

In the present study we show, for the first time, that the hyperalgesic activity associated with type 1 diabetes is clearly manifested in diabetic WT (+/+) C57BL/6 mice and is absent in diabetic BKB_1-R-KO (-/-) mice. The results obtained support our previous findings in CD-1-STZ-mice, on the implication of the inducible BKB_1-R subtype in diabetic hyperalgesia [4,5].

The present results also come in agreement with those provided by Pesquero *et al.*, [6] who demonstrated that in BKB_1-R-KO (-/-) mice, tissue reactions to microbial toxins, local inflammatory agents, and painful thermal and inflammatory stimulations are reduced without any apparent physiological or behavioural impairments.

The hyperalgesic effect observed in WT (+/+) diabetic mice, its potentiation by the selective BKB_1-R agonist DBK, as well as the antihyperalgesic effect of the selective BKB_1-R antagonists, R-715 and R-954, could be sustained by the BKB_1-R-mediated pathway. The BKB_1-R, selectively activated by BKB_1-R agonists, is absent or of little impact under normal physiological conditions [1], whereas, such selective agonists are effective upon over-expression of the BKB_1-R in pathological conditions such as type 1 diabetes. Chronic activation of the inducible BKB_1-R in type 1 diabetes is likely to be amplified by the accumulation of the DBK and other metabolites resulting from the degradation of kinins at the site of inflammation. Thus, $desArg^9$ metabolites may directly induce hyperalgesia by stimulating the inducible BKB_1-R on sensory neurons to release substance P, calcitonin gene-related peptide, neurokinin A and other nociceptive neurotransmitters [1] or by activation of the BKB_1-R induced on cell types (macrophages, fibroblasts or endothelial cells)

with the subsequent release of mediators (prostaglandins, cytokines and nitric oxide) that sensitize the nociceptors [12].

In conclusion, the BKB_1-R subtype appears to mediate the manifestation of hyperalgesia in type 1 diabetic mice. Thus, this receptor emerges as a new target of great potential for the development of specific and selective antagonists directed to reduce the generally excessive responses that are used by the body to counteract noxious stimuli in diabetic patients.

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Fig. 1. Evaluation of hyperalgesia in streptozotocin (STZ)-type 1 diabetes in C57BL/6 wild type (+/+) and BKB_1-R-knockout (-/-) in the hot plate (a) and the tail immersion (b) tests. Diabetes was induced in male C57BL/6 mice using STZ (200 mg/kg, i.p.). On day 7 following STZ injection, nociception was measured by the hot plate and tail immersion tests and the effect of acute i.p. administration of DBK (400 μg/kg), R-715 (400 μg/kg) and R-954 (200 μg/kg), to mice was determined. The hot plate and the tail immersion responses, at 20 min post-drug, for each animal are expressed as mean % of basal latency ± S.E.M. (n=10-14). Values significantly different from saline at *** $P < 0.001$ and from STZ at $^{+++}P < 0.001$.

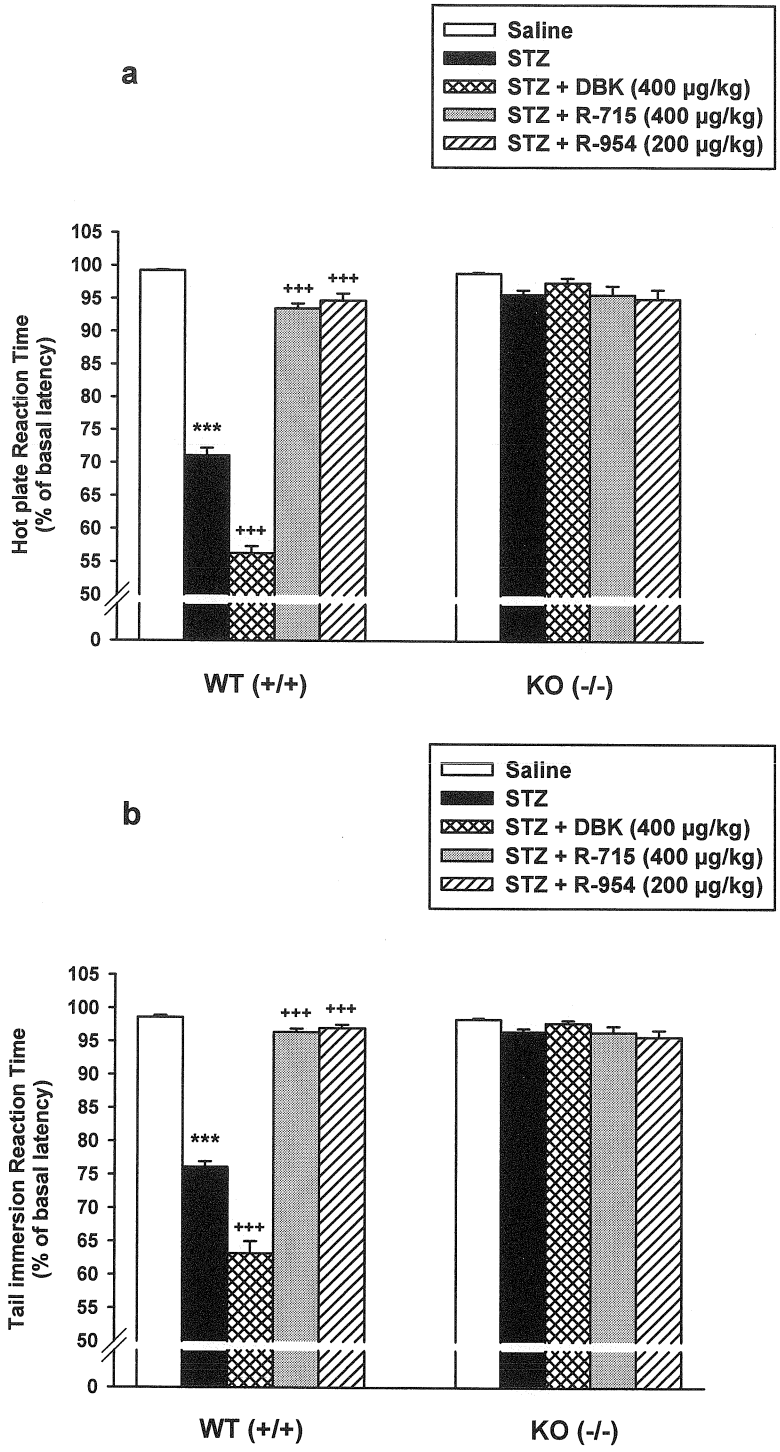

Fig. 1. Evaluation of hyperalgesia in streptozotocin (STZ)-type 1 diabetes in C57BL/6 wild type (+/+) and BKB_1-R-knockout (-/-) in the hot plate (a) and the tail immersion (b) tests.

RESULTS

Article 6

Hyperalgesia in non-obese diabetic (NOD) mice: A role for the inducible bradykinin B_1 receptor

Bichoy H. Gabra, Pierre Sirois

European Journal of Pharmacology 514 (2005) 61-67

Abstract

Most studies performed to investigate the role of the inducible bradykinin B_1 receptor in the pathology and complications of type 1 diabetes have been carried out using the model of streptozotocin (STZ)-induced diabetes. The model of spontaneous autoimmune diabetes in non-obese diabetic (NOD) mice involves a long-term inflammatory process that closely resembles the human type 1 diabetes.

In the present study, we aimed at establishing the correlation between the progress of diabetic hyperalgesia and the incidence of diabetes, as a function of age, in NOD mice. We also evaluated the implication of the bradykinin B_1 receptor, a receptor up-regulated during the inflammatory progress of diabetes, in the development of diabetic hyperalgesia in NOD mice. Female NOD mice were followed up from the 4^{th} to the 32^{nd} week of age for the incidence of diabetes. Only NOD mice with plasma glucose concentration > 20 mmol/l were considered diabetic. The nociception was assessed using the hot plate and the tail immersion pain tests and the effect of acute and chronic administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin and its selective antagonists, R-715 (Ac-Lys-[D-ß Nal^7, Ile^8]desArg^{9}bradykinin) and R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ß Nal^7,ILe^8]desArg^9 bradykinin), on the development of diabetic hyperalgesia was studied.

Diabetic NOD mice developed a significant time-dependent hyperalgesia, as measured in both tests, starting from the 8^{th} week of age with the maximum effect observed over 16 to 20 weeks, whereas the incidence of diabetes in the tested NOD mice was only 40.16 % at the age of 16 weeks and reached a maximum of 73.23 % at the age 24 weeks. Both acute and chronic administration of desArg^{9}bradykinin (400 μg/kg) markedly increased the

hyperalgesic activity in diabetic NOD mice in the hot plate and tail immersion nociceptive tests. The selective bradykinin B_1 receptor antagonist R-715 (400 μg/kg) and its more potent and long acting analogue R-954 (200 μg/kg), administered in acute or chronic manner, significantly attenuated diabetic hyperalgesia in NOD mice in both thermal pain tests and restored nociceptive responses to values observed in control non-diabetic siblings.

Our results bring the first evidence that the development of hyperalgesia in NOD mice, a model of spontaneous type 1 diabetes, precedes the occurrence of hyperglycemia and is mediated by the bradykinin B_1 receptor. It is suggested that bradykinin B_1 receptor antagonism could become a novel therapeutic approach to the treatment of diabetic neuropathic complications.

Keywords: Type 1 diabetes; non-obese diabetic mice; hyperalgesia; kinins; bradykinin B_1 receptor; desArg^{9}bradykinin; R-715; R-954.

1. Introduction

Autoimmune type 1 diabetes is the result of a breakdown of self-tolerance. It is associated with an over-production of cytokines, including interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which leads to T-cell-mediated pancreatic β cell destruction (Hussain et al., 1996; Rabinovitch and Suarez-Pinzon, 1998; Rabinovitch, 1998). These initial events are associated with an inflammatory reaction and the activation of an array of mediators including kinins.

Kinins are key mediators implicated in a variety of biological effects such as cardiovascular homeostasis, inflammation and nociception (Regoli and Barabé, 1981). The bradykinin B_1 receptor, generally silent or absent in healthy states, is induced or activated under pathological conditions including type 1 diabetes, where the over-production of cytokines, the hyperglycemia and the oxidative stress are critical factors for its up-regulation (Marceau et al., 1998; Couture et al., 2001).

Most studies performed to investigate the role of the inducible bradykinin B_1 receptor in the pathology and complications of type 1 diabetes have been carried out using animal models of streptozotocin (STZ)-induced diabetes. Accumulating evidence shows the up-regulation of the bradykinin B_1 receptor subtype in STZ-diabetic animal models. It has been reported that the bradykinin B_1 receptor is over-expressed in the stomach of STZ-diabetic mice since the sensitivity of the stomach fundus to desArg^{9}bradykinin was substantially increased in these animals compared to control non-diabetic mice (Pheng et al., 1997). Lung macrophages and fibroblasts from STZ-diabetic rats express the bradykinin B_1 receptor and their activation leads to the release of cytokines (Koyama et al., 2000). In addition,

the bradykinin B_1 receptor is induced at the peripheral terminals of C-fibres and on the endothelial cells in the lung of STZ-diabetic rats and its activation was shown to be associated with the release of substance P (Vianna et al., 2003). The bradykinin B_1 receptor subtype is also expressed in the kidney and spinal cord of STZ-treated mice (Cloutier and Couture 2000; Mage et al., 2002).

A crucial role has been attributed to the bradykinin B_1 receptor subtype in the development of hyperglycemia and renal abnormalities (Zucollo et al., 1996; 1999; Cantazaro et al., 2004) as well as in altered vascular permeability (Simard et al., 2002) in STZ-induced diabetic animals. Also, we recently demonstrated that STZ-induced diabetes is associated with a marked hyperalgesia in mice, developing 1 week following STZ injection. Such hyperalgesic activity is significantly reduced by both the acute or chronic administration of selective bradykinin B_1 receptor antagonists (Gabra and Sirois, 2002; 2003a; 2003b).

The model of spontaneous autoimmune diabetes in non-obese diabetic (NOD) mice involves a long-term inflammatory process that closely resembles the human type 1 diabetes (Tisch and McDevitt, 1996). It results from a $CD4^+$ and $CD8^+$ T cell-dependent autoimmune process directed against the pancreatic β-cells (Serreze and Leiter, 1994; Tisch and McDevitt, 1996). The major histocompatibility complex (MHC) of the NOD mice (designated $H2^{g7}$) contributes the main component of susceptibility (similar to humans). The MHC class II I-Aβ chain shows the same diabetogenic amino acid substitution (at residue 57) associated with a high risk of development of type 1 diabetes in humans (Atkinson and Leiter, 1999). Non-obese diabetic mice develop inflammation of pancreatic islets (insulitis) at 3 weeks of age, but do not begin to develop diabetes until 10 weeks later (Delovitch and Singh, 1997).

There is sexual dimorphism in the incidence of diabetes in NOD mice in most of the colonies. The disease occurs earlier and more often in females, with an incidence reaching up to 70%, compared to males in which the overall incidence remains below 20% (Fitzpatrick et al., 1991).

The present study aimed at evaluating the development of hyperalgesia in female NOD mice and characterizing the role of the inducible bradykinin B_1 receptor subtype, up-regulated during the inflammatory progress of diabetes, in such a complication.

2. Materials and Methods

2.1. Animals

Female NOD/LtJ mice (Taconic Farms, Inc., Germantown, NY, USA) and female age-matched CD-1 mice (Charles River Breeding Laboratory, St. Constant, PQ, Canada) were used. The mice were housed four by cage with free access to food and water. They were maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10 %) with food and water available ad libitum. Animals were used only once in a given experiment. All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines and were approved by the Animal Care Committee of the University of Sherbrooke.

2.2 Follow-up of diabetes incidence in NOD mice

Mice were screened twice weekly, from 4 to 32 weeks of age, for diabetes onset by measuring urine and plasma glucose concentrations. Urine glucose concentration was determined using reagent strips for urinalysis (Uristix, Bayer Diagnostics, Toronto, ON, Canada). Blood was withdrawn from the retro-orbital sinus of mice with a 50 µl heparinized capillary tube. Plasma glucose concentration was determined with an automatic analyzer (Glucometer Elite XL, Bayer Incorporation, Toronto, ON, Canada) using glucose oxidase / potassium ferricyanide reagents strips. Mice were considered to be diabetic after two consecutive urine glucose concentrations > 5.5 mmol/l and a plasma glucose concentration ≥ 20 mmol/l.

2.3 The hot plate test

A hot plate test (supraspinal pain) derived from that of Eddy and Leimbach (Eddy and Leimbach, 1953) was used. The mouse was placed on a IITC Hot Plate Analgesia Meter (Life Science, CA, USA) adjusted at 55 ± 0.5°C. The hot plate response was the latency observed from the time the mouse was placed on the heated surface until the first overt behavioural sign of nociception such as (i) the mouse licking a hind paw, (ii) vocalization, or (iii) an escape response. The timer was stopped by a foot-operated pedal and the mouse immediately removed from the hot plate. Only mice with basal latency value between 10-15 sec were selected and a maximum cut-off time of 30 sec was observed to avoid excessive pain.

2.4 The tail immersion test

The tail immersion (spinal pain) test was performed according to Coderre and Rollman (1983). The mouse is gently wrapped in a towel, held at a 45° angle to a thermostatically controlled water bath set at 52 ± 1°C. The latency between submersion of the tail and its removal from the water by the animal is recorded, with a maximum cut-off time of 10 sec to avoid excessive pain. Mice with latency value between 2.5-4.0 sec were selected.

2.5. Drugs

DesArg^{9}bradykinin, R-715 (Ac-Lys-[D-ß Nal^7, Ile^8]desArg^{9}bradykinin) (Regoli et al., 2001) and R-954 (Ac-Orn-[Oic^2, α-MePhe^5, D-ß Nal^7, Ile^8]desArg^{9}bradykinin) (Neugebauer et al., 2002) were supplied by IPS Pharma Inc. (Sherbrooke, PQ, Canada).

2.6. Experimental protocol

In a first series of experiments, the development of hyperalgesia in NOD mice was studied. Nociception was assessed once weekly, from 4 to 32 weeks of age using the hot plate and the tail immersion tests.

In a second series of experiments, the effect of acute administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin (400 μg/kg) and its specific antagonists R-715 (400 μg/kg) and R-954 (200 μg/kg) on diabetic hyperalgesia in NOD mice was evaluated. The peptides were administered intraperitoneally (i.p.) to diabetic NOD mice at the age of 24-32 weeks (when the hyperalgesia was maximal and stable). The hot plate and tail immersion latencies were measured, 20 min later.

A final series of experiments was done to study the chronic effects of desArg^{9}bradykinin (400 μg/kg), R-715 (400 μg/kg) and R-954 (200 μg/kg) in diabetic NOD mice. At the age of maximal hyperalgesia, NOD mice were given desArg^{9}bradykinin, R-715 or R-954 i.p., twice daily, for 7 days. By the end of this chronic treatment, the effect of bradykinin B_1 receptor-related peptides was evaluated on nociception using the same thermal pain tests, 5 h following the last dose of the agonist or the antagonist.

NOD mice siblings, which did not become diabetic, were used as control. The selected doses for desArg^{9}bradykinin and R-715 were found to produce maximal effects as previously published (Gabra and Sirois, 2002; 2003a; 2003b). The hot plate and tail immersion responses are presented as % of basal latency.

2.7. Statistical analysis

Data are expressed as mean values ± S.E.M. Analysis of variance (ANOVA) followed by the "Student-Newman-Keuls Multiple Comparisons Test" were performed to assess significance using the Instat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). $P < 0.05$ was considered significant.

3. Results

3.1. Plasma glucose concentration

As illustrated in Fig. 1, female diabetic NOD mice showed a marked age-depended increase in their plasma glucose concentration compared to control non-diabetic siblings. The first significant rise in the plasma glucose concentration was observed in diabetic NOD mice at 11-12 weeks of age and reached 19.58 ± 2.02 mmol/l compared to 5.08 ± 0.45 mmol/l in non-diabetic siblings. The plasma glucose concentration continued to increase with age until it reached a plateau over 24-32 weeks of age (≅ 35 mmol/l).

3.2. Diabetes incidence

The profile of diabetes incidence in diabetic female NOD mice, as a function of age, is shown in Fig. 2. Female NOD mice had an incidence of 5.52 % (7 out of 127 mice) at the age of 12 weeks, but none showed evidence of diabetes before this age as evaluated by glucose concentrations in urine and plasma. They reached an incidence of 40.16 % (51 out of 127 mice) at the 16^{th} week of age, whereas a maximum incidence of 73.23 % (93 out of 127 mice) was observed at 24 weeks of age.

3.3. Correlation between hyperalgesia and diabetes onset

The results showed that diabetic NOD mice develop a significant time-dependent hyperalgesia starting from the 8^{th} week of age with the maximum effect observed over 16 to 20 weeks (Fig. 2). At the age of 8 weeks, the percent of the hot plate basal latency in diabetic NOD mice was 89.37 ± 1.04 versus 99.78 ± 0.29 % in control non-diabetic siblings and the hyperalgesic effect reached its maximum by the 16^{th} week of age when the percent

of the hot plate basal latency was 72.52 ± 0.78% in diabetic NOD mice compared to 98.97 ± 0.18 % in control non-diabetic siblings (n = 48; $P < 0.001$). Similarly, at the age of 8 weeks, the percent of the tail immersion basal latency was significantly decreased (90.22 ± 1.00 %) in diabetic NOD mice compared to non-diabetic siblings (99.75 ± 1.06%). The maximum hyperalgesic effect was observed at 20 weeks of age, when the percent of the tail immersion basal latency in diabetic NOD mice was 66.98 ± 0.54 % compared to 96.87 ± 1.23 % in control non-diabetic siblings (n = 45; $P < 0.001$). The hyperalgesic activity reached a plateau from the 16th to the 32nd week of age in the hot plate test and from the 20th to the 32nd week in the tail immersion test.

It is noteworthy that NOD mice siblings, which did not become diabetic, showed no changes in their response to thermal nociceptive tests over the period from 4 to 32 weeks of age (Fig. 3A and 3B).

3.4. Effects of acute administration of desArg^{9}bradykinin, R-715 and R-954 to NOD mice

The exogenous administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin (400 μg/kg, i.p.) at the age of maximal hyperalgesia (24-32 weeks), markedly increased the hyperalgesic activity observed in diabetic NOD mice in the hot plate (Fig. 3A) and the tail immersion (Fig. 3B) tests. The percent of the hot plate and tail immersion latency compared to the basal latency in desArg^{9}bradykinin-treated diabetic NOD mice was 58.38 ± 1.48 and 55.71 ± 1.95 %, respectively versus 74.13 ± 0.99 and 67.33 ± 1.23 % in untreated diabetic NOD mice (n = 8-10; $P < 0.001$).

Acute administration of the selective bradykinin B_1 receptor antagonists R-715 and R-954 to diabetic NOD mice abolished hyperalgesia and restored nociceptive responses

to values observed in control non-diabetic siblings (Fig. 3A and 3B). R-715 (400 μg/kg, i.p.) restored the % of basal latency to 92.99 ± 1.29 and 89.34 ± 0.73 % and R-954 (200 μg/kg, i.p.) to 90.93 ± 0.43 and 90.91 ± 0.39 % in the hot plate and the tail immersion tests, respectively (n = 8-9; $P < 0.001$).

It is worth mentioning that desArg^{9}bradykinin, R-715 and R-954, tested in control non-diabetic siblings, had no effect at all on the hot plate and tail immersion reaction times (data not shown).

3.5. Effects of chronic administration of desArg^{9}bradykinin, R-715 and R-954 to NOD mice

The chronic administration of desArg^{9}bradykinin, R-715 and R-954 to NOD mice gave similar effects than the acute administration. In brief, the twice daily injection of desArg^{9}bradykinin for 7 days, starting at the age of maximal hyperalgesia (24-32 weeks), increased, whereas that of R-715 and R-954 decreased, hyperalgesia in diabetic NOD mice (Fig. 4A and 4B). The bradykinin B_1 receptor agonist, desArg^{9}bradykinin reduced the percent of the hot plate and tail immersion latency compared to the basal latency to 50.61 ± 1.64 % and 50.51 ± 2.03 %, respectively versus 72.23 ± 1.29 and 70.77 ± 1.46 % in untreated diabetic NOD mice (n = 8-9; $P < 0.001$).

The selective bradykinin B_1 receptor agonist, R-715 significantly increased the percent of hot plate latency to 90.20 ± 0.92 % and the percent of tail immersion latency 89.86 ± 1.23 %, whereas the more potent analogue R-954 increased the hot plate and tail immersion percent latency to 95.04 ± 1.34 and 90.55 ± 1.17%, respectively (n = 8-9; $P < 0.001$).

4. Discussion

In the present study, we showed for the first time the development of a time-dependent hyperalgesia in NOD mice, a model of spontaneous autoimmune type 1 diabetes. We also demonstrated that the observed hyperalgesia does not correlate with the increase in the plasma glucose concentration of the NOD mice, but rather appears very early alongside diabetes and is significant at young age (8-10 weeks), preceding the hyperglycaemic state of the mice. These results are in agreement with our previous findings which showed that STZ-induced diabetes in CD-1 mice is associated with a marked hyperalgesia in thermal nociceptive tests (Gabra and Sirois, 2002; 2003a; 2003b) and suggest that diabetic complications, including hyperalgesia, start to develop during the early inflammatory stages of the disease, even before establishing the hyperglycemia and/or the glucosuria-based diagnosis for diabetes. This could be due to the over-production of cytokines and the oxidative stress developing during the autoimmune response in diabetes as well as the subsequent activation of the mitogen-activated protein kinase (MAP-kinase) and the nuclear transcriptional factor kappa B (NF-κB) pathways (Couture et al., 2001).

Our results confirm the involvement of the inducible bradykinin B_1 receptor subtype in mediating hyperalgesia observed in murine models of type 1 diabetes. Both acute and chronic administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin significantly increased the hyperalgesic effect in diabetic NOD mice, whereas the selective bradykinin B_1 receptor antagonists, R-715 and R-954 were able to abolish such hyperalgesia.

The results obtained also support the previous findings on the expression profile of the bradykinin B_1 receptor in other animal models of type 1 diabetes and the involvement of this

receptor subtype in diabetic complications (Koyama et al., 2000; Simard et al., 2002; Mage et al., 2002; Vianna et al., 2003; Cantazaro et al., 2004). The bradykinin B_1 receptor was recently shown to be up-regulated early in the retina of STZ-diabetic rats, 4 days and up to 21 days, following STZ injection (Abdouh et al., 2003). In the same study, selective bradykinin B_1 receptor agonists evoked relaxation of the retinal vessels and the levels of bradykinin B_1 receptor binding sites remained steady and high over 21 days. Moreover, other studies coming from the same laboratory showed a significant increase in the level of the bradykinin B_1 receptor mRNA expression in the spinal cord and brain of STZ-diabetic rats (2 and 7 days following the injection of STZ) and of its specific binding sites (2, 7 and 21 days following STZ injection) (Ongali et al., 2004). Nevertheless, studies are ongoing in our laboratory to evaluate the profile of expression of the bradykinin B_1 receptor in NOD mice in selected tissues including the spinal cord. Preliminary results obtained show the induction of the bradykinin B_1 receptor in the kidney and the vasculature of the spinal cord of diabetic NOD mice starting from 6-32 weeks of age (data not shown).

The present results are also consistent with those provided by Pesquero et al., (2000) who demonstrated that in bradykinin B_1 receptor-knockout mice, tissue reactions to microbial toxins, local inflammatory agents, and painful thermal and inflammatory stimulations are reduced without any apparent physiological or behavioural impairments. We have also proved that the hyperalgesic activity clearly manifested in type 1 diabetic wild type C57BL/6 mice, is absent in STZ-diabetic bradykinin B_1 receptor-knockout genotype in which desArg^{9}bradykinin has no effect on nociceptive responses (Gabra et al., 2005).

The hyperalgesic effect observed in diabetic NOD mice, its potentiation by the selective bradykinin B_1 receptor agonist desArg^{9}bradykinin, as well as the antihyperalgesic

effect of the selective bradykinin B_1 receptor antagonists, R-715 and R-954, strongly support a role for the bradykinin B_1 receptor in diabetic neuropathy. The bradykinin B_1 receptor subtype, selectively activated by bradykinin B_1 receptor agonists, is absent or of little impact under normal physiological conditions (Couture et al., 2001), but over-expressed in pathological conditions such as diabetes. The up-regulation of the bradykinin B_1 receptor in type 1 diabetes is attributed to several mechanisms including the cytokines (IL-1β and TNF-α)-induced activation of the MAP- kinase and NF-κB pathways (Larrivée et al., 1998; Ni et al., 1998; Schanstra et al., 1998; Zhou et al., 1998; Sardi et al., 1998; Campos et al. 1999). In addition, hyperglycemia and the resulting oxidative stress observed alongside diabetes can activate NF-κB (Yerneni et al., 1999), which is known to induce the bradykinin B_1 receptor (Marceau et al., 1998). Therefore, both the over-production of cytokines and hyperglycemia could trigger the expression of the bradykinin B_1 receptor through NF-κB in diabetes. Moreover, the long-term exposure of the bradykinin B_1 receptor to its endogenous agonist desArg^{9}bradykinin results in increased receptor expression (Faussner et al., 1999). Finally, the bradykinin B_1 receptor was shown to be cross up-regulated by the bradykinin B_2 receptor activation (via autocrine production of cytokines and activation of NF-κB) and/or bradykinin B_2 receptor sensitization (Phagoo et al., 1999).

Chronic activation of the inducible bradykinin B_1 receptor in diabetes is likely to be amplified by the accumulation of desArg^{9}bradykinin and other metabolites resulting from the degradation of kinins at the site of inflammation. Thus, desArg^9 metabolites may directly induce hyperalgesia by stimulating the inducible bradykinin B_1 receptor on sensory neurons to release substance P, calcitonin gene-related peptide, neurokinin A and other nociceptive

neurotransmitters (Couture et al., 2001) or by activation of the bradykinin B_1 receptor induced on selected cell types (macrophages, fibroblasts or endothelial cells) with the subsequent release of mediators (prostaglandins, cytokines and nitric oxide) that sensitize the nociceptors (Dray and Perkins, 1997).

In conclusion, the bradykinin B_1 receptor subtype appears to play a major role in mediating hyperalgesia in type 1 diabetic mice. Thus, this receptor emerges as a new target of great potential for the development of specific and selective antagonists directed to reduce the generally excessive responses that are used by the body to counteract noxious stimuli in diabetic patients.

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Legends for figures

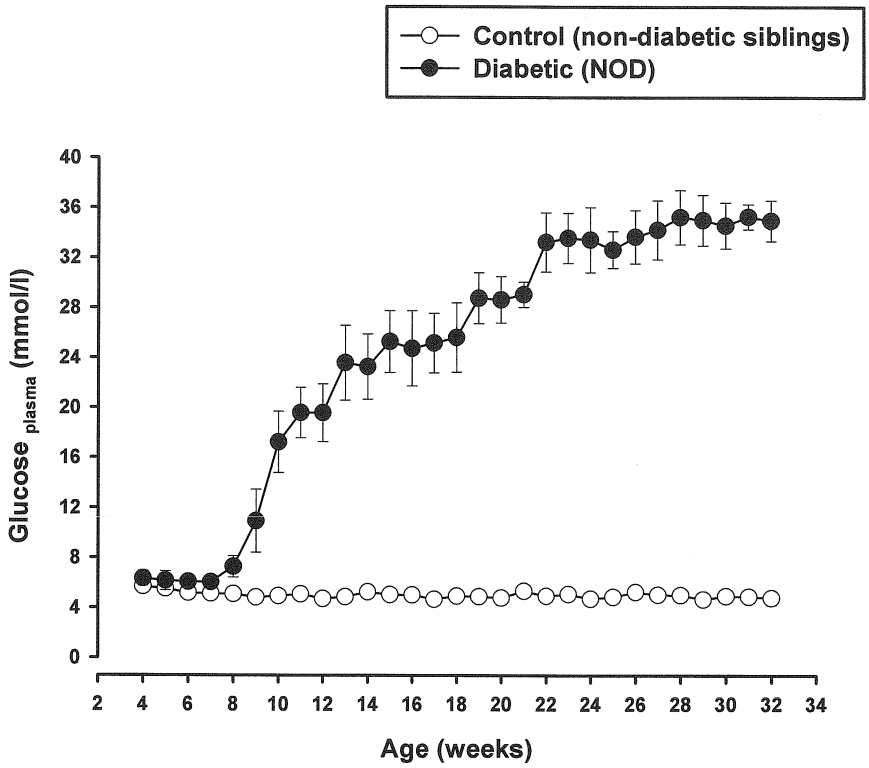
Fig. 1. Plasma glucose concentration in diabetic NOD mice and control non-diabetic siblings. Mice were screened twice weekly, from 4 to 32 weeks of age, for their plasma glucose concentration using an automatic analyzer and were considered diabetic after a plasma glucose concentration ≥ 20 mmol/l. n = 93 diabetic and 34 non-diabetic. Data are expressed as mean plasma glucose concentration ± S.E.M.

Fig. 2. Correlation between the development of hyperalgesia and the onset of diabetes in female diabetic NOD mice. Nociception was assessed once weekly, from 4 to 32 weeks of age using the hot plate and the tail immersion tests. Mice were screened twice weekly for diabetes onset over the same age period by measuring urine and plasma glucose concentrations (n = 93). The hot plate (n = 48) and the tail immersion (n = 45) latencies are expressed as mean % of basal latency ± S.E.M.

Fig. 3. Effects of acute administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin and its selective antagonists, R-715 and R-954 on hyperalgesia in female NOD mice. Nociception was assessed in pre-diabetic (4 weeks of age), control (non-diabetic siblings) and diabetic NOD mice using the hot plate (A) and the tail immersion (B) tests. desAr^{9}bradykinin (400 μg/kg), R-715 (400 μg/kg) and R-954 (200 μg/kg), were given to mice by i.p. injection. The hot plate and the tail immersion latencies were measured 20 min post-treatment. For each animal, data are expressed as mean % of basal latency ± S.E.M.

(n= 8-10). *** values significantly different from control non-diabetic siblings at $P < 0.001$ and $^{+++}$ values significantly different from diabetic NOD mice at $P < 0.001$.

Fig. 4. Effects of chronic administration of the selective bradykinin B_1 receptor agonist, desArg^{9}bradykinin and its selective antagonists, R-715 and R-954 on hyperalgesia in female NOD mice. Nociception was assessed in pre-diabetic (4 weeks of age), control (non-diabetic siblings) and diabetic NOD mice using the hot plate (A) and the tail immersion (B) tests. desArg^{9}bradykinin (400 μg/kg), R-715 (400 μg/kg) and R-954 (200 μg/kg), were administered (twice daily for 7 days) to mice by i.p. injection. The hot plate and the tail immersion latencies were measured 5 h following the last treatment. For each animal, data are expressed as mean % of basal latency ± S.E.M. (n= 8-9). *** values significantly different from control non-diabetic siblings at $P < 0.001$ and $^{+++}$ values significantly different from diabetic NOD mice at $P < 0.001$.



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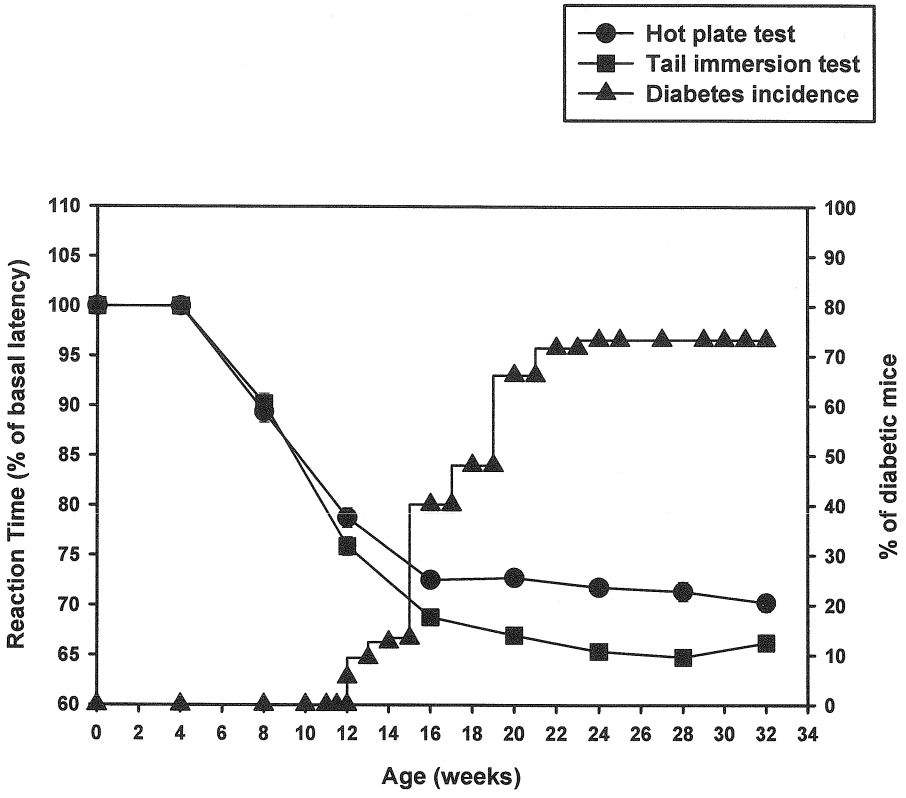

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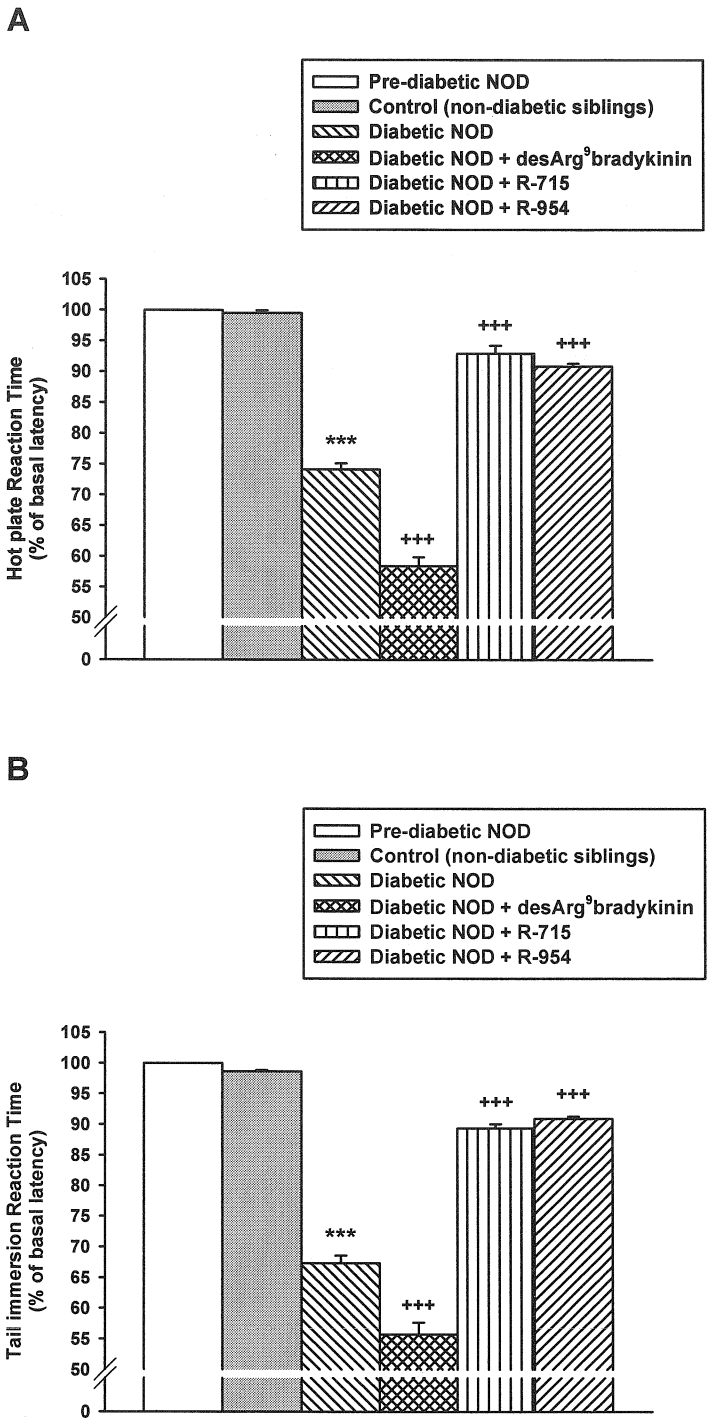

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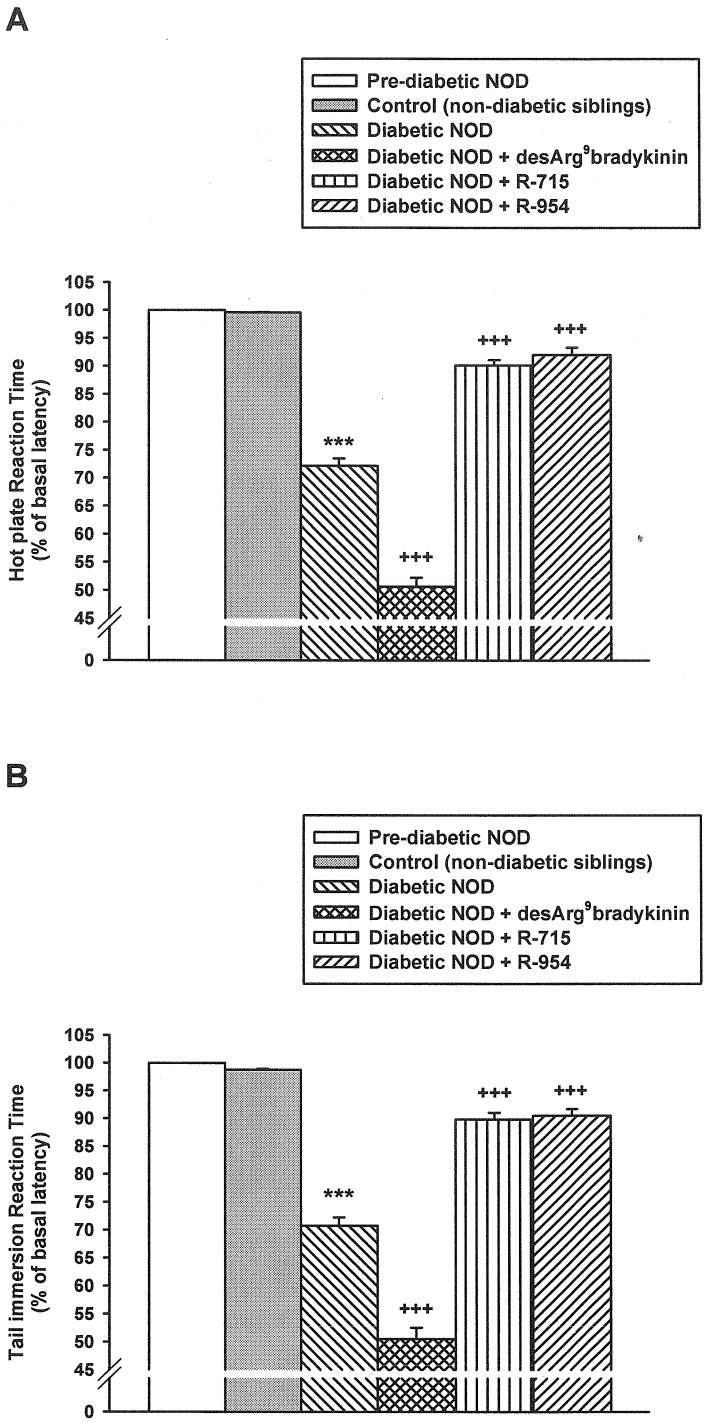

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RESULTS

Article 7

Inhibition of type 1 diabetic hyperalgesia in streptozotocin-induced Wistar versus spontaneous gene-prone BB/Worcester rats: Efficacy of a selective bradykinin B_1 receptor antagonist

Bichoy H. Gabra, Ouhida Benrezzak, Leng-Hong Pheng, Dana Duta, Philippe Daull, Pierre Sirois, François Nantel, and Bruno Battistini

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Abstract

Insulin-dependent type 1 diabetes (T1D) is linked to a series of complications among which, painful diabetic neuropathy (PDN). Several neurovascular systems are activated in T1D including the inducible bradykinin (BK) B_1 receptor (BKB_1-R) subtype. Here, we assessed and compared the efficacy profile of a selective BKB_1-R antagonist on hyperalgesia in two models of T1D: the streptozotocin (STZ)-diabetic Wistar rats and the spontaneous BioBreeding/Worcester-diabetic prone (BB/Wor-DP) rats. Nociception was measured using the hot plate test. The STZ-diabetic rats developed stable hyperalgesia (35% decrease in their hot plate reaction time) from 1-4 weeks post-injection. The BB/Wor-DP rats developed hyperalgesia over time that preceded hyperglycemia, starting at the age of 6 weeks (9% decrease in the hot plate reaction time) and stabilizing over the age of 16-24 weeks to a maximum (60% decrease in the hot plate reaction time). Single acute s.c. administration of the selective BKB_1-R antagonist induced a significant time- and dose-dependent attenuation of hyperalgesia in both STZ-diabetic and BB/Wor-DP rats. Thus selective antagonism of the inducible BKB_1-R subtype may constitute a novel and potential therapeutic approach for the treatment of PDN.

Key Words: Type 1 diabetes, hyperalgesia, streptozotocin, BB/Wor rats, bradykinin B_1 receptor

Introduction

Insulin-dependent type 1 diabetes (T1D) is a chronic metabolic disorder characterized mainly by hyperglycemia, dyslipidemia and increased vascular permeability, which lead to generalized vasculopathy affecting the kidneys, the eyes, the heart and nerves (nephropathy, retinopathy, cardiomyopathy and neuropathy) (1).

Painful diabetic neuropathy (PDN) is generally considered to be one of the most troublesome and common complications of diabetes, affecting nearly 30-50% of all diabetic patients at one stage of their life (2). Patient with PDN suffer from burning, stabbing and lancinating pain, tingling, in addition to developing hyperalgesia and/or allodynia (2, 4). PDN remains very difficult to prevent and very difficult to treat. Although several drugs (anticonvulsants, antidepressants) have been tried to ameliorate neuropathic pain in diabetic patients, their effectiveness is mitigated and associated with a number of side effects (5). To assess novel, mechanism-based, therapeutic approaches against PDN, one needs adequate animal models.

Various animal models of T1D have been developed and used to study the etiology of diabetes and its related complications for some time (6), especially the widely used streptozotocin (STZ)-induced model of T1D known to ablate pancreatic β-cells and to induce severe, but mostly non-lethal insulin-deficient diabetes (7), a wasting disease with high blood cytokine levels (8). This model reflects similar symptoms to those observed in diabetic patients with PDN, by developing tactile allodynia (9, 10) and hyperalgesia to mechanical (11 - 14), thermal (15, 16) and chemical stimuli (17). However, this chemically-induced model of T1D remains constantly hyperglycemic. Therefore, many observations based on the STZ models have an uncertain significance for the complications of diabetes in modern-day

practice, since the forms of diabetes that the physicians treat today are no longer wasting diseases (8). To further evaluate potential effective therapies against PDN, as well as to elucidate the mechanisms of pain, other models need to be used in comparison.

The BioBreeding/Worcester (BB/Wor) rat (Wistar-derived) provides a spontaneous diabetic prone (DP) autoimmune model of T1D, closely resembling humans (18, 19), in which the susceptibility to diabetes is linked to the major histocompatibilty complex (MHC) $RT1^u$ (20). Key features include abrupt onset of insulin-dependent, ketosis-prone diabetes between 60 and 120 days of age (21) and lymphocytic insulitis (occurring before and during the acute phase of hyperglycemia) with virtually complete destruction of the pancreatic β-cells (22). This model also reflects similar symptoms to those observed in diabetic patients with neuropathy and have been used to define neuropathy-functional abnormalities (23, 24).

One novel approach aimed at alleviating the symptoms of PDN involving the modulation of an inducible receptor of the kinin system, the bradykinin (BK) B_1 receptor (BKB_1-R) subtype (25). The kinin system regulates, in part, cardiovascular homeostasis, nociception and the acute phase of inflammation via the constitutive bradykinin B_2 receptor subtype and participates in chronic inflammatory and nociceptive processes through its BKB_1-R subtype (26).

Current experimental evidence suggests that diabetes up-regulates the BKB_1-R as a consequence of the over-production of inflammatory cytokines and the oxidative stress in response to the state of hyperglycemia (27, 28). In addition, selective BKB_1-R antagonists were shown to (i) attenuate renal abnormalities including increased urine volume and increased excretion of protein, nitrite and kallikrein (29, 30), (ii) decrease vascular permeability changes in the liver, pancreas, duodenum, ileum, spleen, heart, kidney

and stomach in STZ-diabetic mice (31) and in the skin and retina of STZ-diabetic rats (32), and (iii) alleviate diabetic hyperalgesia in STZ-mice as measured via thermal nociceptive tests (33 - 35).

In the present study, we aimed at evaluating the development of diabetic hyperalgesia in Wistar STZ-diabetic and age-matched non-diabetic controls as well as in BB/Wor-DP and BB/Wor diabetic resistant (DR) (BB/Wor-DR) rats, in addition to characterizing the anti-hyperalgesic effect of a selective BKB_1-R antagonist, R-954 (36). We found that both models of T1D developed hyperalgesia that was effectively inhibited by the treatment with the BKB_1-R antagonist.

Materials and Methods

Animals

Male Wistar rats (8 weeks of age; 180-200g) were purchased from Charles Rivers (St. Constant, QC, Canada), whereas male BB/Wor-DR and BB/Wor-DP rats were purchased from Biomedical Research Models Inc. (Springfield, MA, USA). The rats were housed four by cage and maintained under conditions of standard lighting, (alternating 12-h light/dark cycle), temperature (22 ± 0.5°C) and humidity (60 ± 10%). Wistar rats had free access to normal Purina rat chow diet # 5075 (Charles River; St-Constant, QC, Canada) and tap water, whereas BB/Wor rats had free access to enriched Autoclavable diet # 7012 (Harlan TEKLAM; Madison, WI, USA) and sterilized water (37). All experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines and were approved by the Animal Care Committee of the University of Sherbrooke.

Induction of type 1 diabetes in Wistar rats

Insulin-dependent T1D was induced in Wistar rats, at 9 weeks of age, by injecting a single intra-peritoneal dose of STZ (65 mg/kg) (38). Age-matched controls were injected with an equal volume of saline. The induction of diabetes was confirmed by measuring the circulating plasma concentration of glucose obtained from tail vein blood samples, 4 days post-STZ injection, via an automatic analyzer (Glucometer Elite XL, Bayer Incorporation, Toronto, ON, Canada) using glucose oxidase / potassium ferricyanide reagents strips. Diabetic animals used in our study had a plasma glucose concentration higher than

30 mmol/L, while the normal value is from 5-7 mmol/L. The rate of induction of diabetes was 89%.

Follow-up of diabetes in BB/Wor rats

Both strains of BB/Wor rats were screened twice a week, from 4 to 24 weeks of age, to asses the onset of diabetes through urine and circulating plasma concentrations of glucose. Urine concentrations of glucose were determined using reagent strips for urinalysis (Uristix, Bayer Diagnostics, Toronto, ON, Canada). Plasma concentrations of glucose were measured as previously described. The BB/Wor-DP rats were considered diabetic after two consecutive urine glucose values > 5.5 mmol/L and a plasma glucose concentration ≥ 20 mmol/L, and were immediately treated once daily with a long-acting insulin (PZI 40; 0.9 μl per 100 g body weight, subcutaneously (s.c.)) in order to control glucosuria, ketonuria, limit weight and muscle loss and prevent death (39).

Assessment of nociception

Hyperalgesia, as a nociceptive response, was assessed in both control and diabetic rats using the hot plate test. Hyperalgesia reflects an exaggerated response to noxious stimuli evoked by a sensitization of peripheral nociceptors in addition to a central facilitation of pain transmission at the level of the dorsal horn neurons and thalamus. It is worthy to mention that in our previous published studies, the STZ-diabetic (33 -35) as well as the non-obese diabetic (40) mice showed no difference in their hyperalgesia evaluated by both spinal (tail immersion and tail flick) and supra-spinal (hot plate and plantar stimulation) pain tests.

These observations generated within and published from our laboratories are reassuring since the literature is somewhat confusing over the effects of diabetes on thermal latencies which may be due in part to methodological details such as the use of different species, limbs, methods of heat application, degree of temperature and age-related diabetic status (41).

The hot plate test

A hot plate test derived from that of Eddy & Leimbach (42) was used. The rat was placed inside a plexiglass box (25x25x38 cm) in order to be confined to the anodized heated surface of the IITC Hot Plate Analgesia Meter (Life Science, Woodland Hills, CA, USA) adjusted at 50 ± 0.5°C. The hot plate response was defined as the latency observed from the time the rat was placed on the heated surface until the first overt behavioural sign of nociception: (i) fanning or licking a hind paw, (ii) vocalization, or (iii) an escape response such as jumping. The timer was stopped by a foot-operated pedal and the rat was immediately removed from the hot plate. A maximum cut-off time of 30 sec was observed to avoid excessive pain. A pre-treatment basal latency was assessed 3 separte times over 3 consecutive days in all groups of rats (controls and diabetic) at various ages and time of treatment. A mean was calculated to establish a stable pre-drug response latency (see below for protocol).

Experimental protocol

The nociceptive response and the development of hyperalgesia were assessed by the hot plate test in all groups of rats. STZ-diabetic and age-matched control rats were studied over a period of 28 days following STZ administration, on days 7, 14, 21 and 28. In a

second set, BB/Wor-DP and age-matched BB/Wor-DR rats were studied weekly from 4 to 24 weeks of age.

Once a stable degree of hyperalgesia was established in diabetic rats (at 7 days post-STZ and over 18-22 weeks of age in BB/Wor-DP rats, the acute effect of a selective BKB_1-R antagonist, R-954, on nociception was evaluated at various time-points over 48 h post-s.c. administration of R-954 or saline.

The following groups, each of 9-14 rats, were used and administered saline or various doses of the selective BKB_1-R antagonist: (i) control non-diabetic + saline; (ii) control non-diabetic + R-954 (3 mg/kg); (iii) STZ-diabetic + saline; (iv) STZ-diabetic + R-954 (4 doses 0.3, 1, 2 and 3 mg/kg); (v) BB/Wor-DR + saline; (vi) BB/Wor-DR + R-954 (3 mg/kg, s.c.); (vii) BB/Wor-DP + saline; (viii) BB/Wor-DP + R-954 (4 doses: 0.3, 1, 2 and 3 mg/kg).

Pharmacokinetics of the BKB_1-R antagonist

Circulating plasma concentrations of the BKB_1-R antagonist, R-954, were measured over 24 h in a separate set of experiments using four groups of chronically instrumented, conscious and unrestrained rats: control and STZ-Wistar rats, and BB/Wor-DR and BB/Wor-DP rats. Animals were injected s.c. with either saline or R-954 (2 and 3 mg/kg, bolus). Arterial blood was collected from 5 min to 24 h after dosing for the determination of circulating plasma concentrations of the drug.

Arterial plasma concentrations were measured using a two-solvent gradient separating system [A: HPLC grade water (0.05% TFA); B: acetonitrile (0.025% TFA); flow rate: 1.0 ml/min; gradient at 0 min, 5% B; 2-25 min, 5-50% B; 50-95 min, 26-30% B

and 35-40 min, 95-5% B] with a Zorbax 300SB-C18 analytical column (4.6 x 150 mm, 5 μm) on an HPLC system (Agilent model 1100 series). The detection was performed using a diode array detector (Agilent model DE292916850). Plasma concentrations of lower doses (0.1, 0.3 and 1 mg/kg) of the BKB_1-R was calculated using Winnonlin first order two compartmental PK model simulation (Pharsight) (43, 44).

Drugs

Streptozotocin (Zanosar™; Pharmacia & Upjohn Inc.; Mississauga, ON, Canada) was freshly dissolved in saline and immediately administered to Wistar rats. Insulin PZI 40 (Biomedical Research Models Inc.; Springfield, MA, USA) was given to BB/Wor DP rats. The selective BKB_1-R antagonist, R-954, (Ac-Orn-[Oic^2, α-Me Phe^5, D-β Nal ^{7}, Ile ^{8}] desArg^9 BK (BKB_1-R Ki = 2.4 nmol/L; BKB_2-R Ki > 10 μmol/L) (36) was synthesized at IPS Pharma Inc. (Sherbrooke, QC, Canada) and dissolved in sterile saline prior administration to all groups of rats.

Statistical Analysis

All data are presented as means ± SEM. Statistical analysis was performed using the Student's *t*-test for unpaired data or the analysis of variance (ANOVA) followed by the "Student-Newman-Keuls Multiple Comparisons Test" were performed to assess significance using the Instat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). A probability (p) less than 0.05 was considered significant.

Results

Glycemia and growth in Wistar and BB/Wor rats

Administration of a single dose of STZ (65 mg/kg, i.p.) increased glycemia by 3.8-fold, from 6.7 ± 0.4 mmol/L to 25.3 ± 4.8 mmol/L, 4 days post-STZ. Chronic hyperglycemia remained elevated and stable from 1 to 4 weeks post-STZ (Table 1).

The circulating plasma concentration of glucose remained stable at 5.08 ± 0.45 mmol/L in BB/Wor-DR rats from 4 - 24 weeks of age (Table 1). Conversely, BB/Wor-DP rats developed an age-dependent increase in their blood glucose level. Once BB/Wor-DP rats showed two consecutive urine glucose values > 5.5 mmol/L and a plasma glucose concentration ≥ 20 mmol/L, they were immediately injected s.c. with insulin (once daily) to survive, which normalized their glycemia (Table 1). Ten to thirty percent of BB/Wor-DP rats became diabetic by the age of 8-10 weeks. The incidence reached 58% at 16 weeks and 74% at 24 weeks of age (Fig. 1B).

The growth curve and body weight of STZ-rats were affected by the development of diabetes. At the time of saline or STZ-injection (9 weeks of age), rats weighted 208 ± 8.1 and 202 ± 11.7 g, respectively and their body weight increased by >20 and 10%, to 261 ± 7.3 and 233 ± 10.3 g, respectively, 1 week post-injection. Both the non-diabetic controls and the STZ-diabetic rats continued rats continued to gain weight from age 10-13 weeks (1 to 4 weeks post-injection of saline or STZ). The weight gain was significantly higher in control non-diabetic (52%) versus hyperglycemic STZ-diabetic rats (29%) at 4 weeks post-saline or STZ injection, respectively. In comparison, the growth curves of BB/Wor-DR versus insulin-treated BB/wor-DP rats were similar and showed a 4.9- and 5.7-fold increase, respectively, from the age of 4 to 24 weeks (85.9 ± 6.7 and 71.4 ± 3.1 to 420.0 ± 4.3 and 409.3 ± 3.9 g, respectively).

Diabetes-induced thermal hyperalgesia in STZ-diabetic and BB/Wor-DP rats

Hyperalgesia developed within 1 week in STZ-diabetic rats and remained significantly elevated and stable over 4 weeks (Fig. 1A). The hot plate reaction time in the STZ-treated group was 7.5 ± 0.3; 7.3 ± 0.5; 6.9 ± 0.9 and 7.9 ± 0.6 s compared to 10.6 ± 0.3; 11.0 ± 0.4; 10.5 ± 0.3 and 10.9 ± 0.4 s in the control-saline group at week 1 through 4, respectively, thus a 28-34% faster reaction time ($p < 0.001$; Fig. 1A).

Hyperalgesia also developed in BB/Wor-DP rats compared to BB/Wor-DR rats (Fig. 1B). At the age of 4-5 weeks, both BB/Wor-DR and BB/Wor-DP rats had similar nociceptive responses. The BB/Wor-DP rats developed a significant age-dependent hyperalgesia starting at 6 weeks of age with the maximum effect observed over 16 to 20 weeks (Fig. 3B). At the age of 6 weeks, the hot plate reaction time in BB/Wor-DP rats decreased by 20%, from 15.3 ± 0.3 in BB/Wor-DR rats to 12.2 ± 0.3 sec in BB/Wor-DP rats ($p < 0.001$). The maximal hyperalgesic effect was observed at 16 weeks of age when the hot plate reaction time decreased by 64%, from 12.6 ± 0.3 in BB/Wor-DR rats to 6.1 ± 0.1 s in BB/Wor-DP rats ($p < 0.001$). The hyperalgesic activity stabilized from 16 weeks of age up to 24 weeks (Fig. 1B). Noticeably, nociception, measured thrice a week, was affected by aging in BB/Wor-DR rats, slowly but steadily decreasing by 19%, from 15.4 ± 0.4 to 12.4 ± 0.8 sec from 4 to 10 weeks of age, and remained stable thereafter for up to 24 weeks of age (Fig. 1B).

Correlation between hyperalgesia and diabetes onset in BB/Wor-DP rats

The onset of hyperalgesia in BB/Wor-DP rats did not parallel the onset of T1D as determined by the first signs of hyperglycemia (Fig. 1B). The BB/Wor-DP rats showed a 5.3% incidence of diabetes by 8-10 weeks of age, as evaluated by urine and plasma concentrations of glucose, and already a 43-55% decrease in hot plate reaction time compared

to their baseline at 4 weeks of age (Fig. 1B). The incidence of T1D in BB/Wor-DP rats reached 58% at 16 weeks of age, whereas hyperalgesia was already maximal (idem up to 24 weeks) with a 64% decrease in time latency compared to their baseline at 4 weeks of age (Fig. 1B).

Effects of the BKB_1-R antagonist on diabetic hyperalgesia

Acute s.c. administration of the highest effective dose of the selective BKB_1-R antagonist, R-954, (3 mg/kg) did not affect the reaction time in the hot plate test in control Wistar (Fig. 2A) and in BB/Wor-DR (Fig. 2B) rats.

In contrast, treatment of STZ (Fig. 2C) and BB/Wor-DP rats (Fig. 2D) with the selective BKB_1-R antagonist, R-954, (0.3, 1, 2, 3 mg/kg) mediated a significant time- and dose-dependent inhibition of hyperalgesia.

In STZ-diabetic rats, the maximal transient effect was observed at 3-4 h post-injection, for the 4 tested doses, and inhibition reached 75-100% ($p < 0.001$). The highest dose (3 mg/kg) was totally effective within 1 h and an 80% inhibition remained after 24 h before the effect disappeared at 48 h post-treatment. The other three doses were ineffective after 4-6 h (0.3 and 1 mg/kg) and 8-9 h (2 mg/kg) (Fig. 2C). The two highest doses even caused a transient hypoalgesia (higher than baseline at 10.3 ± 0.4 s measured in control non-diabetic rats) (Fig. 2C).

In BB/Wor-DP rats, treatment with the BKB_1-R antagonist also decreased hyperalgesia although it appeared to be less effective (Fig. 2D). The degree of hyperalgesia was higher in BB/Wor-DP than in STZ-daibetic rats (Δ of 49% versus 34% compared to respective baseline in control rats; Fig. 1A). Maximal inhibition was observed over 2 to 4 h in all groups following the acute administration of the BKB_1-R antagonist (Fig. 2D). A 98% inhibition was

observed at the highest dose of 3 mg/kg (back to normal values in BB/Wor-DR control rats), whereas lower doses (0.3, 1 and 2 mg/kg) significantly attenuated (33, 49 and 89%, respectively) the diabetes-mediated hyperalgesia. The hot plate reaction time increased to 7.8 ± 0.2; 8.8 ± 0.4 and 10.7 ± 0.6 compared to 6.4 ± 0.1 s in untreated BB/Wor-DP rats at doses of 0.3, 1 and 2 mg/kg, respectively ($p < 0.001$). The anti-hyperalgesic effect was sustained for up to 24 h (with a 50% inhibition) at the highest dose of R-954 (3 mg/kg), whereas the dose of 2 mg/kg was effective (35% inhibition) up to 7 h post-treatment (Fig. 2D). The inhibitory effects disappeared 5 h following administration of the two lowest doses.

Pharmacokinetic profile of the BKB_1-R antagonist in STZ-diabetic rats

Circulating plasma concentrations of the selective BKB_1-R antagonist were evaluated in the STZ-diabetic rats treated at doses of 2 and 3 mg/kg (Fig. 3A). R-954, revealed a prolonged coverage provided by a single s.c. administration at a dose of 3 mg/kg, reaching 0.09 ± 0.08 mg/L within 15 min and up to 0.67 ± 0.10 mg/L at 2 h post-injection. Plasma concentrations of lower doses (0.1, 0.3, and 1mg/kg) of R-954 (Fig. 3B) were calculated using Winnonlin first order two compartmental PK model simulation (Pharsight). The simulation suggests that Cmax plasma levels may reach 0.04, 0.12, 0.41, 0.80 and 1.2 mg/L at the 0.1, 0.3, 1, 2, and 3 mg/kg doses respectively. Therefore, the minimal effective plasma concentration of R-954 would be about 100 nM.

Discussion

The present study compared two rat models of T1D, one chemically-induced and the other gene-prone, in the incidence of hyperalgesia in parallel to the onset of the disease and the glycemic status over time. In addition, both models were used to assess the role of the kinin system at mediating supra-spinal thermal nociception, or pain, via the inducible BKB_1-R subtype.

Insulin-dependent T1D developed in both rat models as previously described (18, 19, 37). We observed that both groups of diabetic rats developed thermal hyperalgesia to similar extent but under different glycemic states. Hyperalgesia was apparently more elevated in insulin-treated BB/Wor-DP rats (60% reduction in their latency) than in the non-treated STZ rats (35%). The nociceptive response of healthy non-diabetic Wistar rats did not change with age over 4 weeks post-STZ injection (between 10 and 13 weeks of age). Conversely, the hot plate reaction time decreased by 20% in BB/Wor-DR rats (between 4 and 10 weeks of age) and remained stable thereafter for up to 24 weeks. Such change in nociceptive response could be attributed to the aging process of the BB/Wor-DR sub-strain, but could also occur in control Wistar rats over the same timeline (between 4 and 10 weeks of age), which was not covered in this study.

Thermal hyperalgesia was previously demonstrated in STZ-induced T1D rats under a steady state of hyperglycemia (7, 15, 16). It remains to be established if insulin-treated STZ-diabetic rats would still develop the same degree of hyperalgesia. However, ongoing studies from our laboratory show no effect for insulin on reversing or attenuating thermal hyperalgesia in STZ-diabetic mice (data not shown). Thermal and mechanical hyperalgesia were also reported in BB/Wor-DR versus BB/Wor-DP rats (23, 45) at various ages.

In the present set of experiments, we show that the nociception threshold of BB/Wor-DR rats is affected by aging, stabilizing at 16 weeks of age (adulthood). Furthermore, we report that the observed diabetic hyperalgesia in BB/Wor-DP rats does not correlate with the increase in their plasma glucose concentration, but rather manifests very early alongside diabetes and is significant at young age (6-8 weeks of age), preceding the hyperglycaemic state of the mice. These results are in agreement with our previous findings in NOD mice (38) and suggest that diabetic complications including hyperalgesia could start to develop during the early inflammatory stages of the disease, even before establishing the hyperglycemia and/or the glucosuria-based diagnosis for diabetes. This could be due to the over-production of cytokines and the oxidative stress developing during the autoimmune response in diabetes as well as the subsequent activation of the mitogen-activated protein kinase (MAP-kinase) and the nuclear transcriptional factor kappa B (NF-κB) pathways (26).

Acute treatment of diabetic rats with a selective BKB_1-R antagonist induced a significant time- and dose-dependent attenuation of supra-spinal thermal hyperalgesia, even restoring, for a time, the nociceptive responses to values observed in control non-diabetic animals. Such treatment did not affect normal healthy rats. These results are consistent with previous results obtained in our laboratories using the STZ murine model (33 - 35), and further confirmed in BKB_1-R knockout mice where STZ-diabetic mice were not hyperalgesic in addition to the lack of effect of a selective BKB_1-R agonist, $desArg^9BK$ (DBK) upon enhancing hyperalgesia as in wild-type mice (46). The DBK was reported to induce thermal hyperalgesia in wild-type mice but not in BKB_1-R knockout mice (47). It was also shown to enhance the degree of hyperalgesia in STZ diabetic mice (33 - 35).

The occurrence of the inducible BKB_1-R subtype has been also clearly established in the present (diabetes; 48 - 50) and other pro-inflammatory conditions (pain (26); arthritis (51); allergic asthma, (52)) via the action of various cytokines (23; 53; 54), chemokines, growth factors and reactive oxygen species-NF-κB-mediated pathways, all of which may even contribute directly to central and peripheral neuropathic pain and hyperalgesia (55, 56).

Chronic activation of the inducible BKB_1-R in diabetes is likely to be amplified by the accumulation of DBK and other metabolites resulting from the degradation of kinins at the site of inflammation. Thus, $desArg^9$ metabolites may directly induce hyperalgesia by stimulating the inducible BKB_1-R on sensory neurons to release substance P, calcitonin gene-related peptide, neurokinin A and other nociceptive neurotransmitters (26) or by activation of the BKB_1-R induced on selected cell types (macrophages, fibroblasts or endothelial cells) with the subsequent release of mediators (prostaglandins, cytokines and nitric oxide) that sensitize the nociceptors (57). Afferent neurons (mainly C and Aδ fibres) innervating the skin are know to act as vasodilators neurons controlling the blood flow, the release of vasoactive peptides and the cutaneous vascular permeability. Under diabetic inflammatory conditions, plasma protein leakage induced by immune mediators including kinins, can stimulate afferent nerve fibres and enhance their excitability.

The duration of the inhibitory effect of the pseudo-peptidic BKB_1-R antagonist, R-954, was directly related to the dosage (low dose: 8 hrs; highest dose: 24 hrs) based on the present route of administration (s.c.). However, the duration off effect at the dose of 3 mg/kg was much longer than one would expect from the pharmacokinetics data. This could be explained either through differential compartmentalization of the drug at the site of action

versus plasma or that the off-rate of the drug bound to the BKB_1-R is longer than its plasma clearance.

It is believed that many factors, through other mechanisms, may play an important pathogenic role in nociceptive sensory neuropathy (substance P, calcitonin gene related peptide, C-peptide and neurokinin (58, 59) opening a wide range of potential targets for therapeutic intervention. We observed that selective antagonism of the inducible BKB_1-R subtype is effective in two distinct pre-clinical animal models of T1D developing experimental neuropathy, one of which presenting hyperalgesia prior to hyperglycemia. Hyperalgesia remains in insulin-treated normoglycemic conditions. Thus, the antagonism of the inducible BKB_1-R subtype may constitute a novel therapeutic target against painful neuropathy in type 1 diabetic patients. It remains to be established if type 2 diabetic models, also developing hyperalgesia, would be improved via BKB_1-R antagonism. Since selective BKB_1-R antagonists were also reported to inhibit nociception in other non-diabetic models (60), the participation of the kinin system through this inducible receptor may become more relevant in pain and analgesia.

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	Glucose $_{[plasma]}$ (mmol/L)	
Time (weeks post-injection)	**Control**	**STZ-diabetic**
1	6.70 ± 0.40	26.54 ± 4.80
2	5.98 ± 0.66	28.54 ± 2.15
3	6.41 ± 0.85	27.56 ± 3.21
4	5.54 ± 0.98	28.96 ± 2.79
Age (weeks)	**BB/Wor-DR**	**BB/Wor-DP**
4	5.85 ± 0.15	5.65 ± 0.09
8	5.28 ± 0.06	5.31 ± 0.20
12	4.87 ± 0.08	5.79 ± 0.74
16	5.50 ± 0.36	5.69 ± 0.89
20	4.80 ± 0.18	4.69 ± 0.87
24	4.75 ± 0.15	4.40 ± 0.65

Table 1. Circulating plasma concentrations of glucose in control non-diabetic compared to STZ-diabetic rats over 1 to 4 weeks post-saline or STZ injection and in BB/Wor-DR compared to insulin-treated BB/Wor-DP rats over 4 to 24 weeks of age. Wistar rats were injected with saline or STZ at 9 weeks of age. BB/Wor-DP rats were treated with insulin upon the onset of diabetes up-to 24 weeks of age Data are presented as means ± SEM (n=9-14).

Legends for figures

Fig. 1. Incidence of hyperalgesia, measured as the time of reaction in the hot plate test in age-matched **(A)** control-saline (open circle) compared to STZ-diabetic rats (black square) rats and in **(B)** BB/Wor-DR (open circle) compared to insulin-treated BB/Wor-DP (back square) rats over 4 to 24 weeks of age. The incidence of type 1 diabetes (black diamond) is confirmed by two consecutive urine glucose values > 5.5 mmol/L and a plasma glucose concentration ≥ 20 mmol/L, prior to the administration of exogenous insulin. Data are presented as means ± SEM (n = 9-14).

Fig. 2. Effects of the selective BKB_1-R antagonist, R-954, on hyperalgesia measured as the time of reaction in the hot plate test in **(A)** control-saline Wistar rats (open circle; + R954 at 3 mg/kg, s.c.; open triangle), **(B)** BB/Wor-DR rats (open circle; + R-954 at 3 mg/kg, s.c.; open triangle), **(C)** STZ-diabetic rats (black square), and **(D)** BB/Wor-DP (black square) treated with R-954 at 0.3 (inverted open triangle), 1 (black circle), 2 (open diamond) and 3 (black triangle) mg/kg, over 48 hr post-s.c. bolus injection. Data are presented as means ± SEM (n = 9-14).

Fig 3. (A) Plasma concentrations of R-954 in STZ-diabetic rats treated with R-954 at 2 (open circle) and 3 mg/kg (black circle). Data are presented as means ± SEM (n = 4-6). **(B)** Simulation of plasma concentration of R-954 in STZ-diabetic rats treated with doses of 0.1 to 3 mg/kg calculated using Winnonlin first order two compartmental pharmacokinetics model simulation (Pharsight).

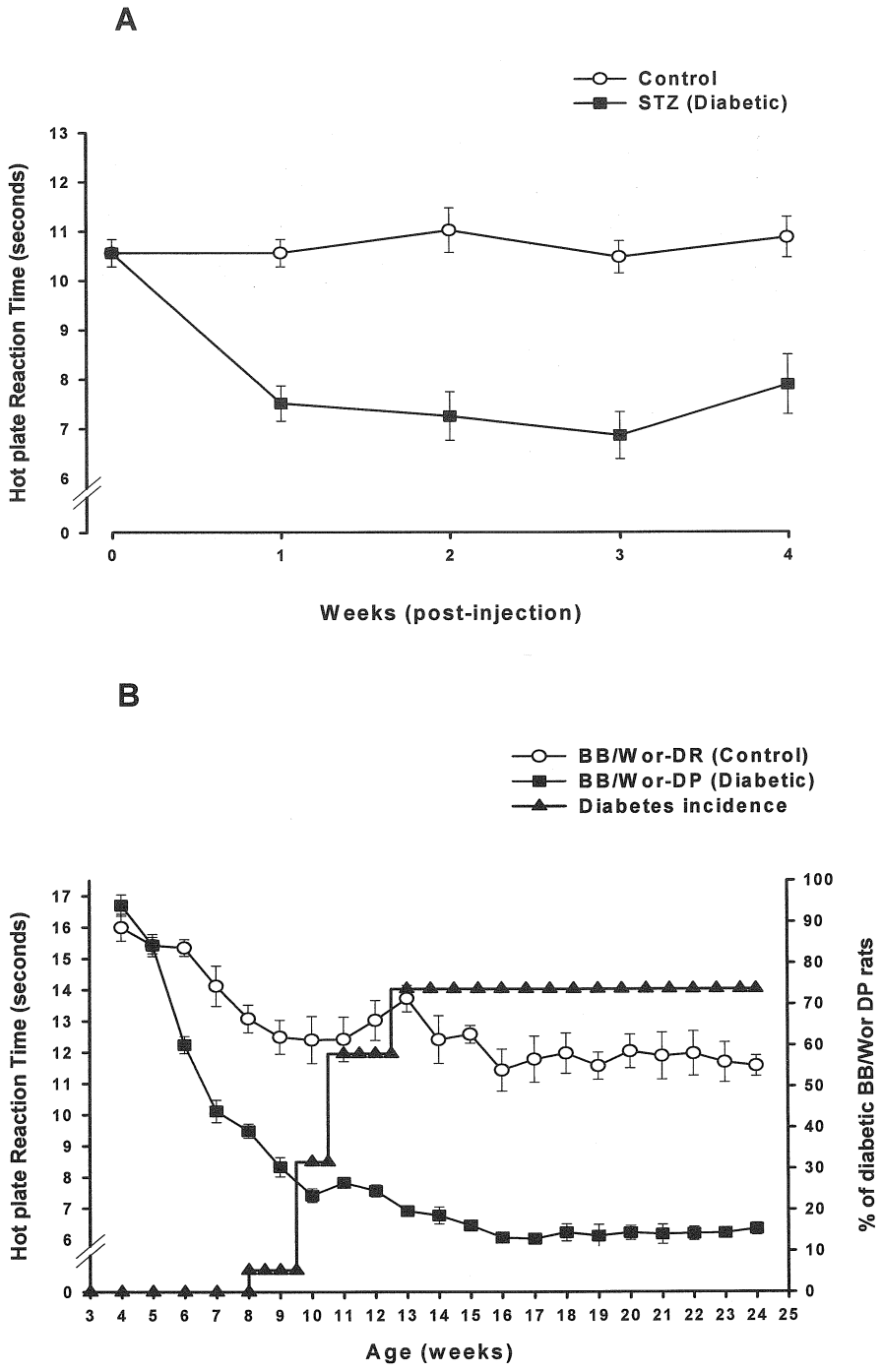


Fig. 1. Incidence of hyperalgesia, measured as the time of reaction in the hot plate test in age-matched (A) control-saline (open circle) compared to STZ-diabetic rats (black square) rats and in (B) BB/Wor-DR (open circle) compared to insulin-treated BB/Wor-DP (back square) rats.

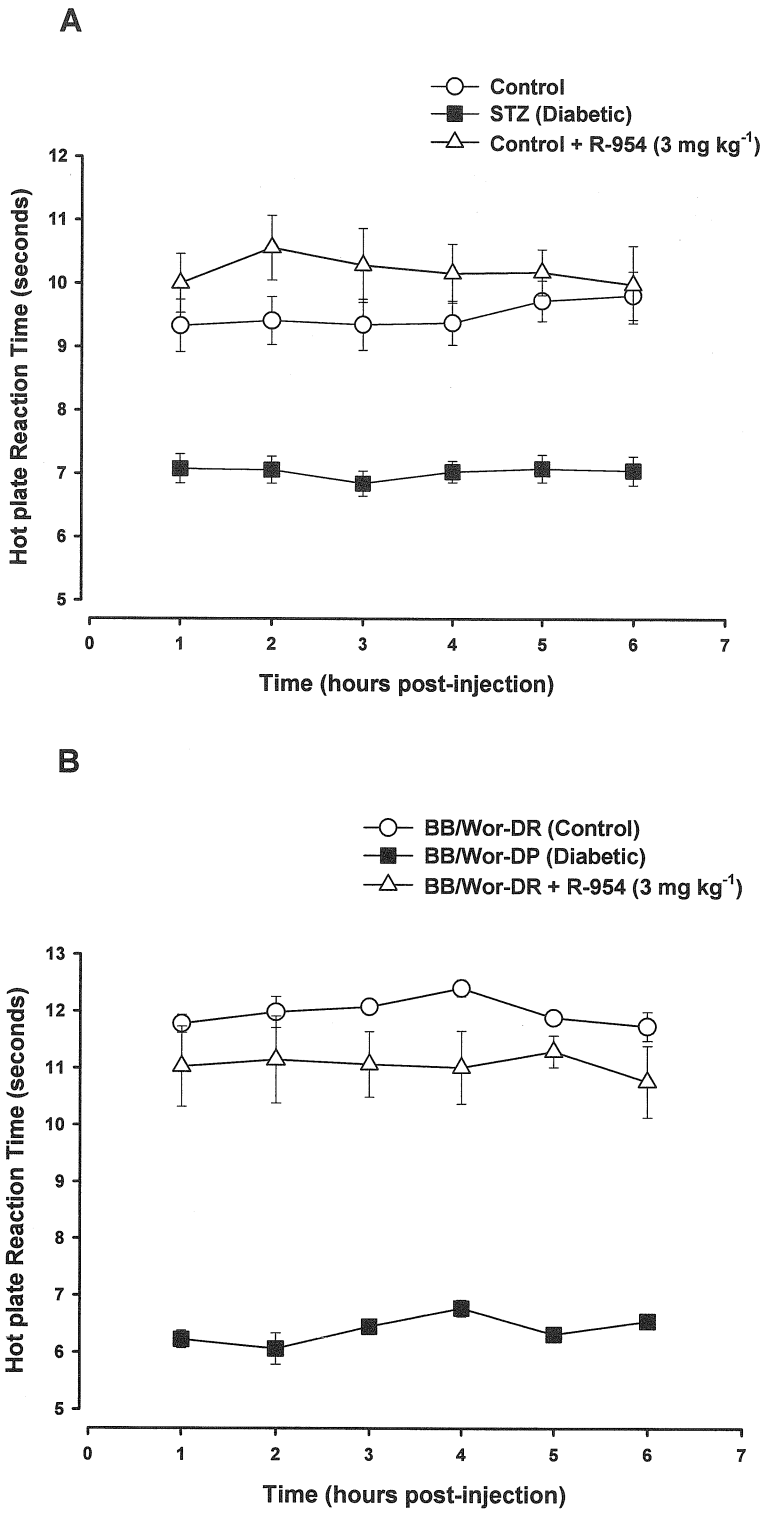


Fig. 2. Effects of the selective BKB_1-R antagonist, R-954, on hyperalgesia in (A) control-saline Wistar rats and (B) BB/Wor-DR rats.

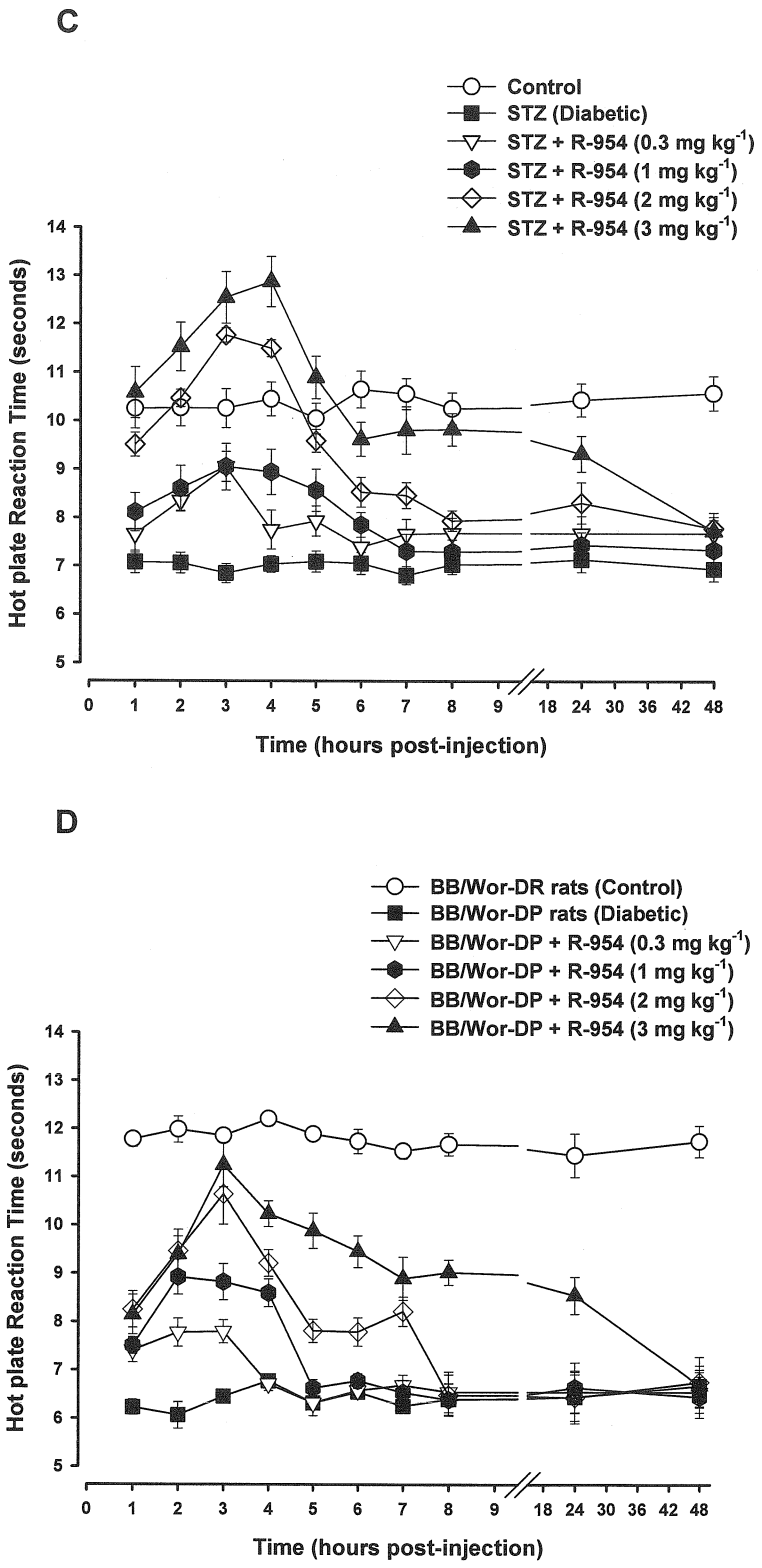


Fig. 2. Effects of the selective BKB_1-R antagonist, R-954, on hyperalgesia in (C) STZ-diabetic rats and (D) BB/Wor-DP rats.

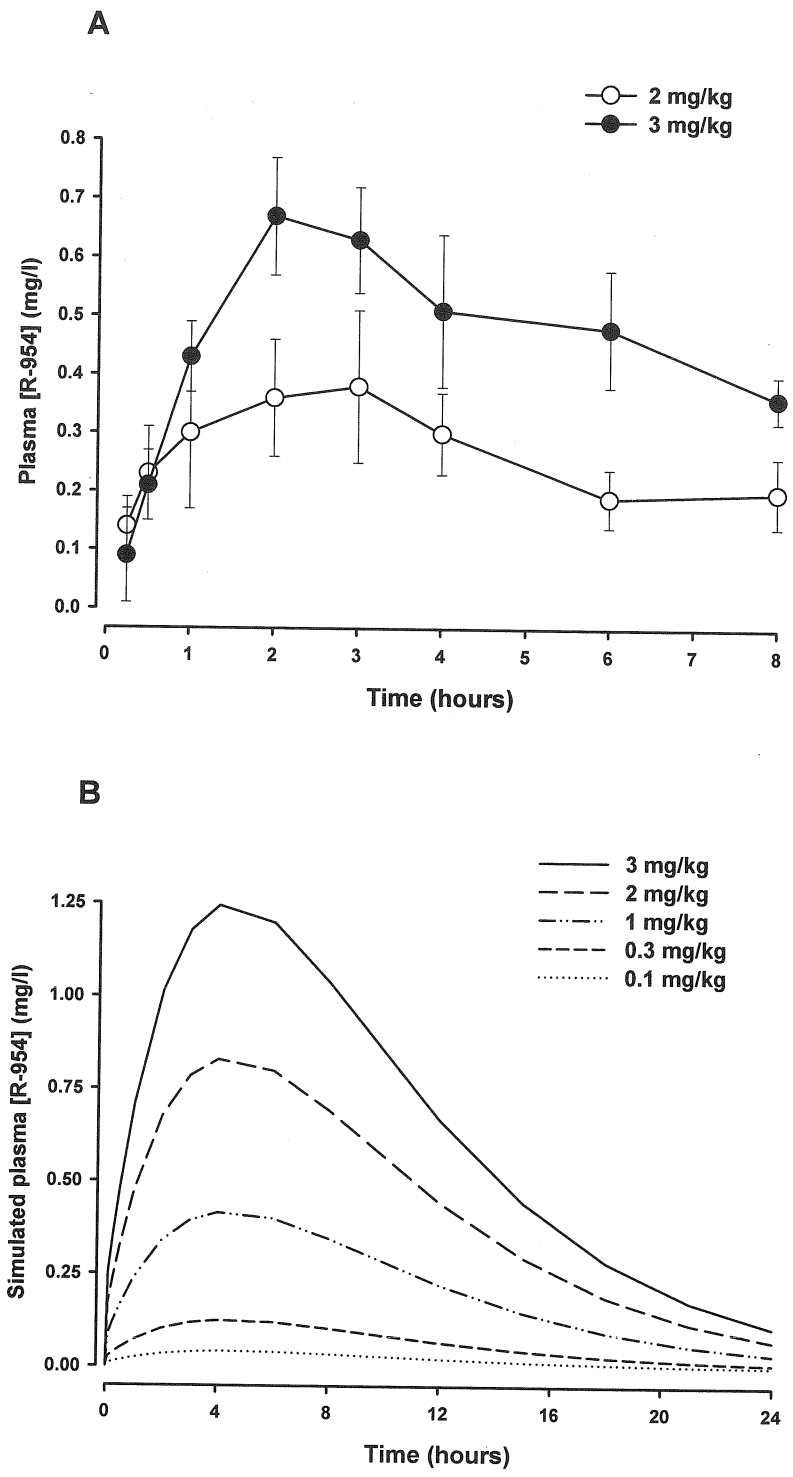


Fig 3. (A) Plasma concentrations of R-954 in STZ-diabetic rats treated. (B) Simulation of plasma concentration of R-954 in STZ-diabetic rats.

ADDITIONAL RESULTS

1. Physiological markers of type 1 diabetes

1.1. Methods

A part of our project aimed at evaluating the physiological parameters of type 1 diabetes in STZ-diabetic male Wistar rats and in BB/Wor-DP rats. Circulating arterial blood samples were collected in heparin-lithium vacutainers from control versus STZ-diabetic rats at time 0, 1 and 4 weeks post-saline or -STZ injection. Plasma concentrations of glucose (mmol/l), cholesterol (mmol/l) and triglycerides (mmol/l) were measured using a Vitros 950 from Beckman Coulter Inc (Hialeah, FL, USA) through UV/visible colorimetric assays, whereas insulin (ng/ml) was measured with a commercially available rat insulin kit (S-1238; ELIS7536) from Peninsula Laboratories Inc. (San Carlos, CA, USA). The consumption of drinking water (ml/day) and food (g/day) was also determined.

1.2. Results

1.2.1. Physiological markers of type 1 diabetes in STZ-diabetic Wistar rats

Single treatment of Wistar rats with STZ (65 mg/kg, bolus i.p.) produced (a) a marked hyperglycemia, (b) hypoinsulinemia, (c) hypercholesterolemia, (d) hypertriglyceridemia, (e) polydipsia and (f) polyphagia (Figure 21). The mean plasma glucose concentration, measured 1 week post-STZ administration, was reported at 29.75 ± 3.60 mmol/l and was stable (3.5-fold elevation) for up to 4 weeks (30.66 ± 4.60 mmol/l) in diabetic rats compared to 7.57 ± 0.37 mmol/l in normal healthy rats along the same time. Insulin secretion was almost abolished (96% decrease) after 1 and up to 4 weeks post-STZ injection, from 2.94 ± 0.88 to 0.12 ± 0.08 ng/ml.

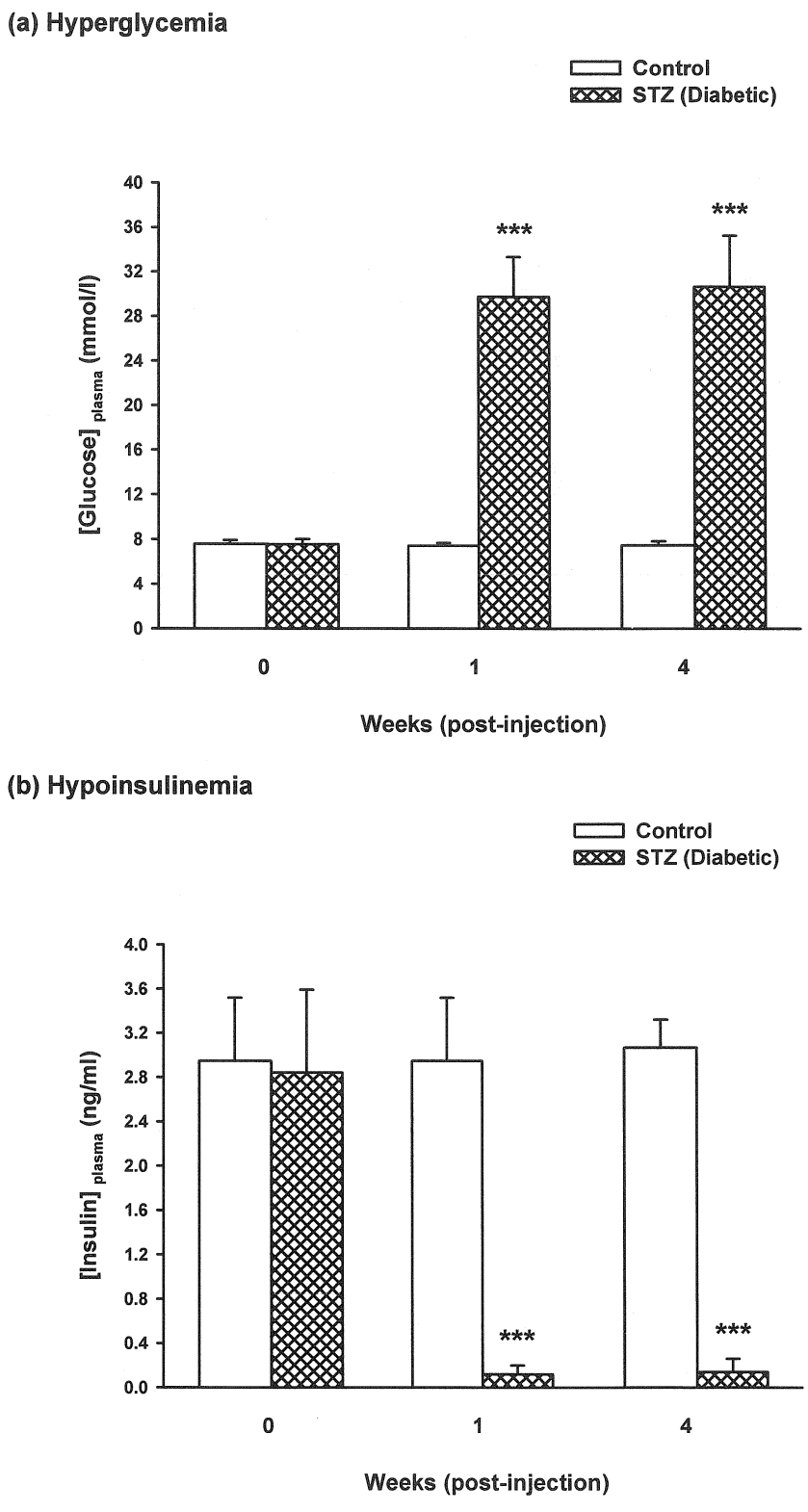


Figure 21. Physiological markers of type 1 diabetes in STZ-diabetic Wistar rats.

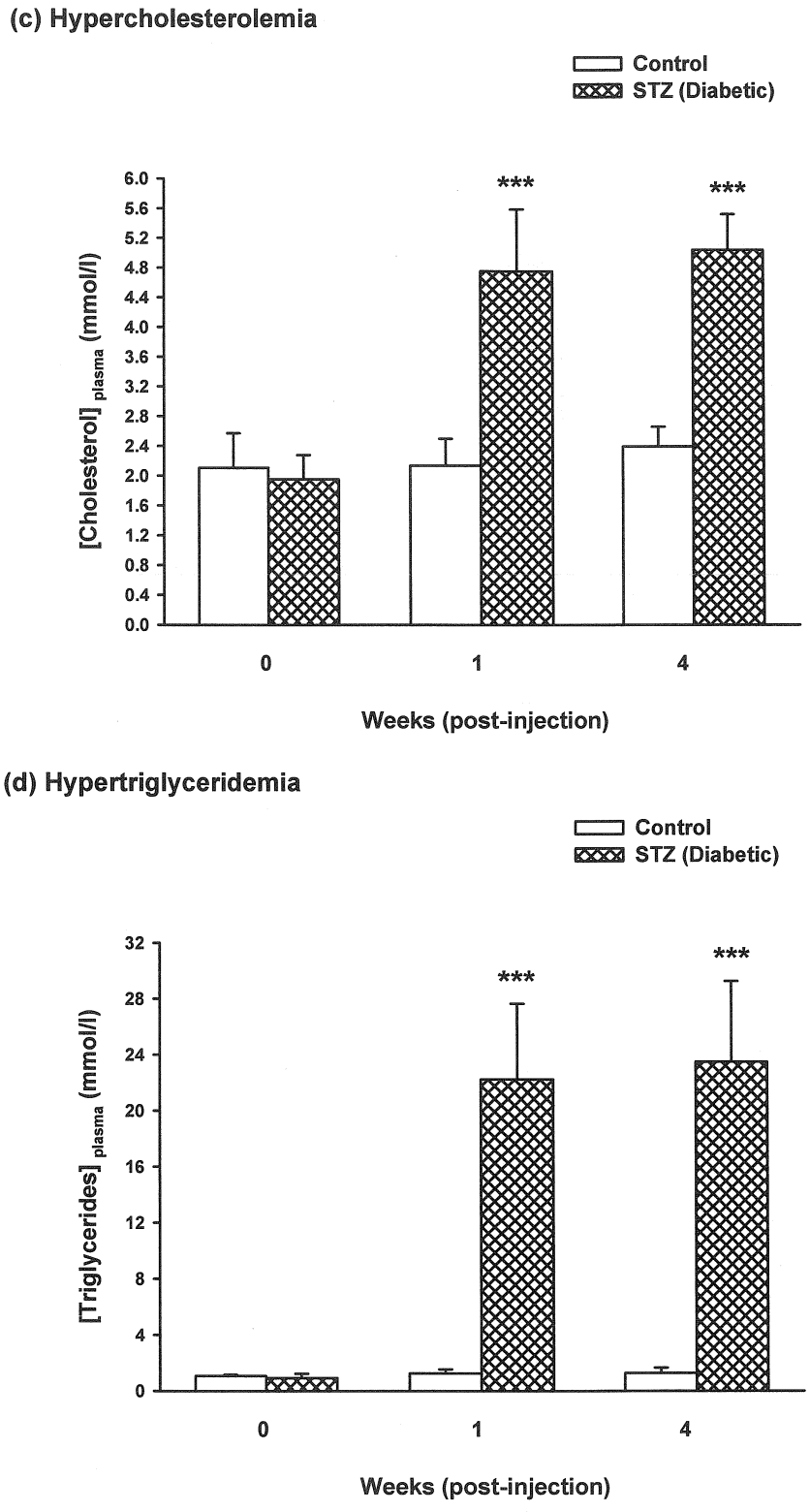
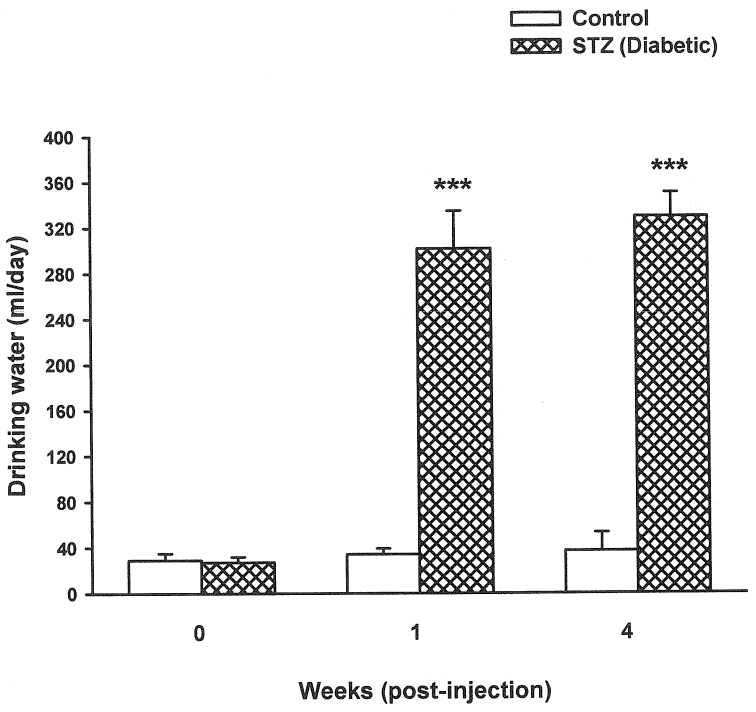


Figure 21. Physiological markers of type 1 diabetes in STZ-diabetic Wistar rats.

(e) Polydipsia



(f) Polyphagia

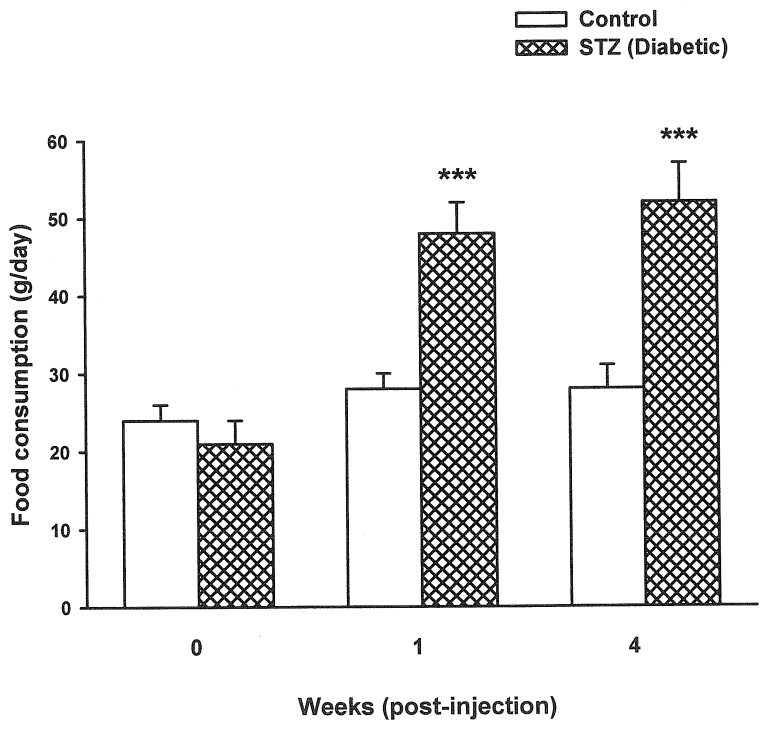


Figure 21. Physiological markers of type 1 diabetes in STZ-diabetic Wistar rats.

Both cholesterol and triglycerides increased from 1.95 ± 0.32 to 4.75 ± 0.84 (2.5-fold) and from 0.93 ± 0.29 to 22.22 ± 5.41 mmol/l (24-fold), respectively after 1 week from STZ administration, and remained steadily elevated (2.6- and 25-fold) at 4 weeks post-treatment. Diabetic rats drank and ate much more (7.5- and 8.6-fold, and 1.4- and 1.8-fold), 1 and 4 weeks post-STZ, respectively, than age-matched controls (LAWSON *et al.*, 2005a).

1.2.2. Physiological markers of type 1 diabetes in BB/Wor-DP rats

The STZ-induced model of type 1 diabetes remains constantly hyperglycemic; therefore, some observations based on this model might have an uncertain significance for the complications of diabetes. In another part of our project we used the spontaneous gene-prone BB/Wor rat model in comparison. The BB/Wor rat should be well hydrated, free of ketosis, gaining body weight, and maintained in a moderate state of glucosuria to avoid hypoglycemia. The circulating plasma concentration of glucose remained stable at 5.08 ± 0.45 mmol/l in BB/Wor-diabetic resistant (BB/Wor-DR) rats from 4 - 24 weeks of age (Figure 22). Conversely, BB/Wor-diabetic prone (BB/Wor-DP) rats developed a time-dependent increase in their glycemia. Once diagnosed as diabetics, BB/Wor rats were immediately treated daily with a single s.c. injection of a long-acting insulin (PZI 40; 0.9 µl per 100 g body weight) in order to control (i) glucosuria, (ii) ketonuria, (iii) limit weight and muscle loss and (iv) prevent death; upon the onset of diabetes (between 8-10 weeks) till the end of the study at 24 weeks of age. The growth curves for BB/Wor-DR versus insulin-treated BB/wor-DP rats were similar and showed a 4.9- and 5.7-fold increase, respectively, from the age of 4 to 24 weeks. In addition, the treatment with insulin normalized cholesterolemia, triglyceridemia, food and water consumption in BB/Wor-DP rats (Figure 22).

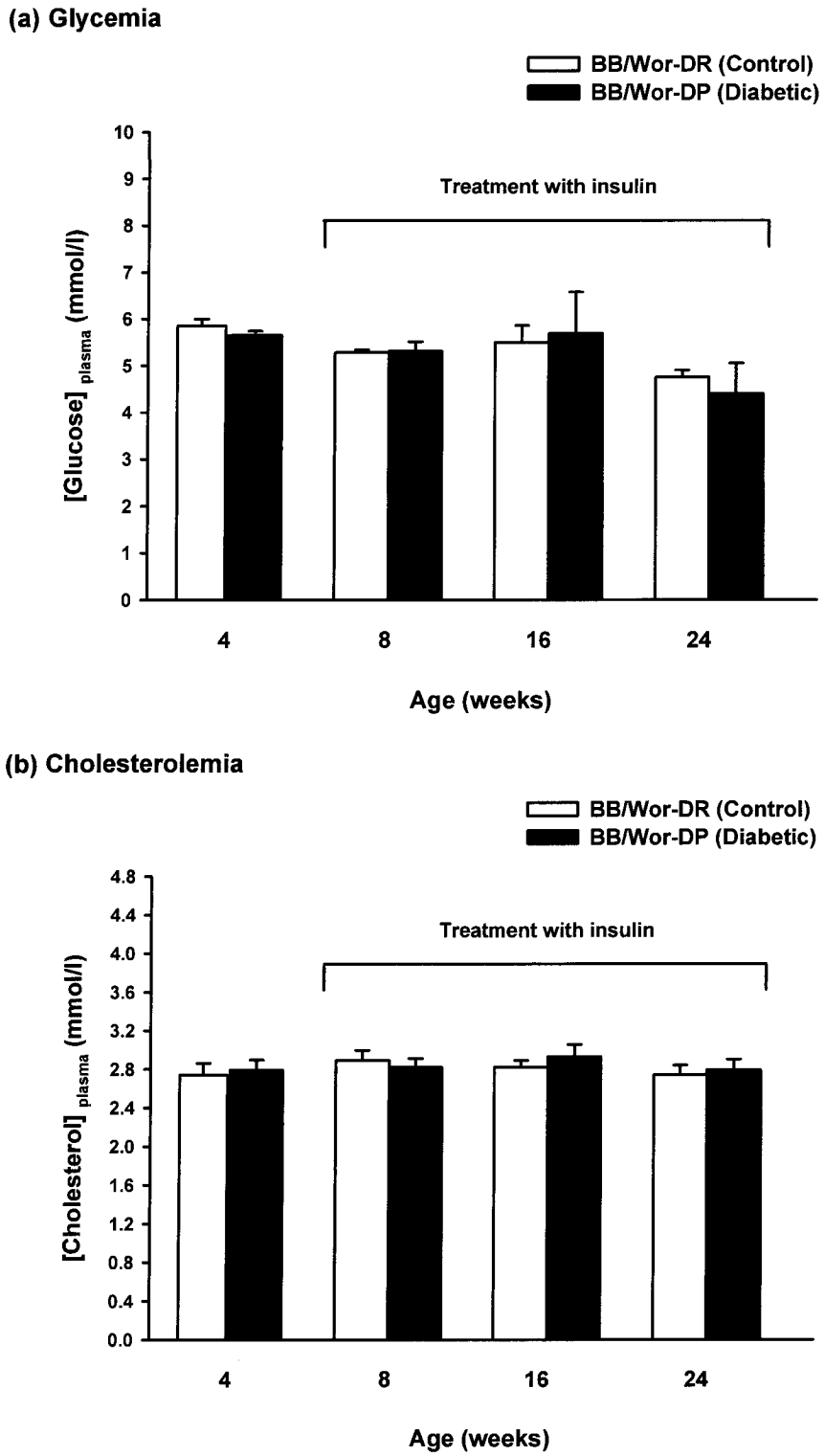


Figure 22. Physiological markers of type 1 diabetes in BB/Wor-DP rats.

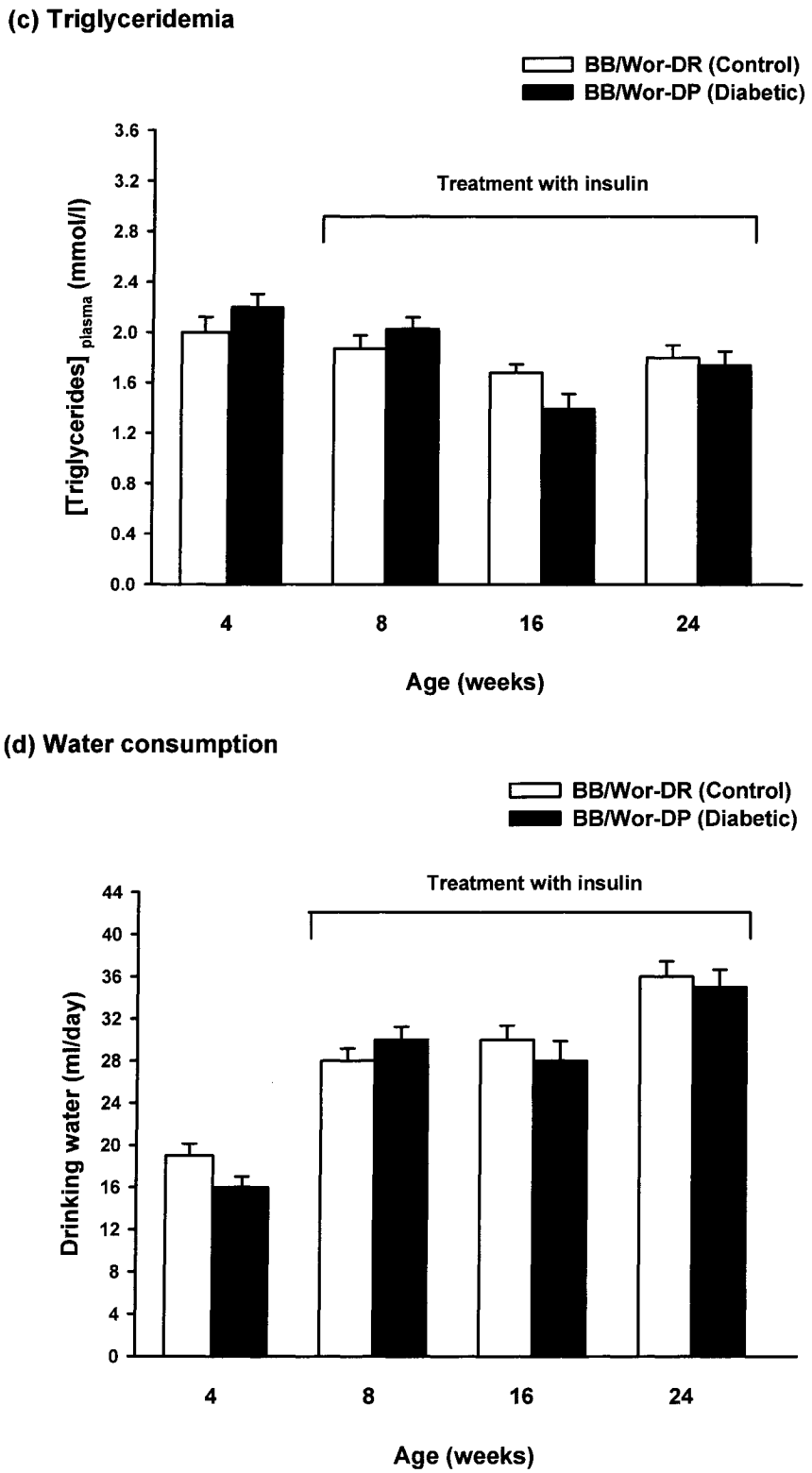


Figure 22. Physiological markers of type 1 diabetes in BB/Wor-DP rats.

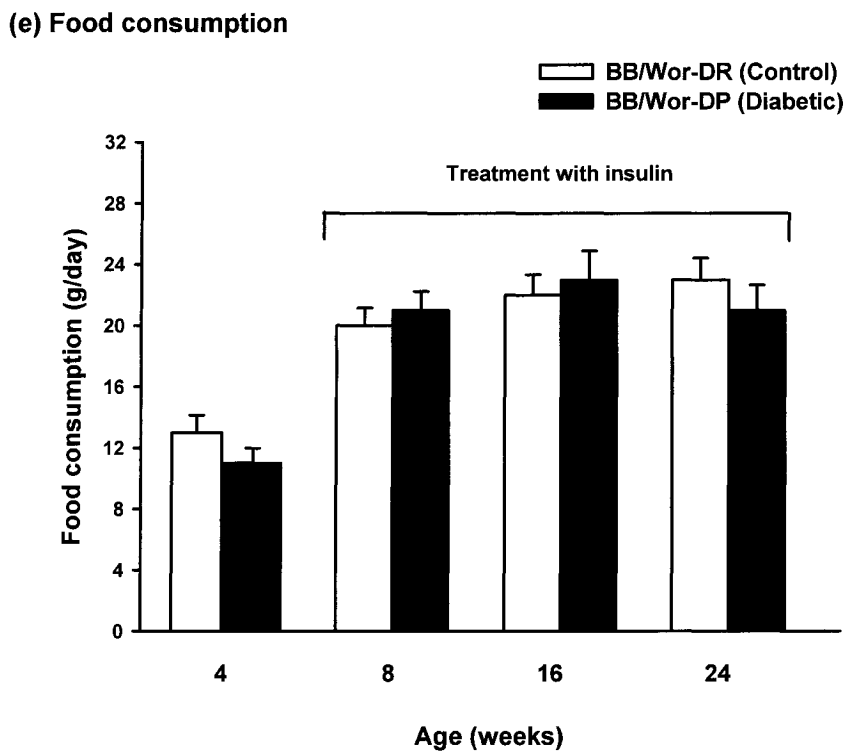


Figure 22. Physiological markers of type 1 diabetes in BB/Wor-DP rats.

2. Expression profile of BKB_1-R in diabetes

2.1. Methods and results

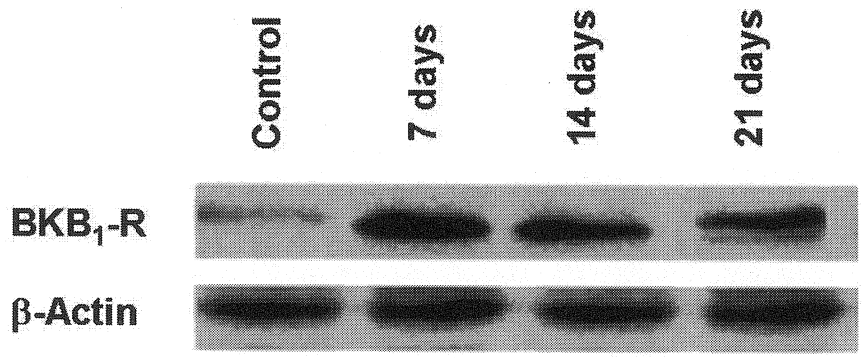
Another goal of our study was to evaluate the expression of the BKB_1-R subtype in animal models of type 1 diabetes. Our results have shown a significant up-regulation of the BKB_1-R in the pancreas of STZ-mice (7, 14 and 21 days following STZ administration) as demonstrated by Western Blot analysis (using anti-BKB_1-R antibody) and by determining the quantitative changes in the specific BKB_1-R mRNA by Real-Time (RT) polymerase chain reaction (PCR) in a Lightcycler (Roche, Mannheim, Germany) (Figure 23) (study done in collaboration with Dr Orlando Catanzaro, Department of Physiology, Faculty of Pharmacy and Biochemistry, Buenos Aires, Argentina).

We also showed by immunohistochemical techniques as described by LAMONTAGNE *et al.*, (2001), an up-regulation of the BKB_1-R in the retina and choroid tissues (Figure 24) as well as in the blood vessels of skin in the STZ-diabetic Wistar rats (Figure 25) using a specific BKB_1-R antibody (sc-15045; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and 3,3'Diaminobenzidine (DAB) substrate kit as detecting reagent (brown colour; Biogenex, San Ramon, CA, USA)

Finally, we evaluated, by immunochemistry, the profile of expression of the BKB_1-R in NOD mice in selected tissues including the kidney and spinal cord. Our results have demonstrated the induction of the BKB_1-R on the papillary ducts and collecting tubules of the kidney (Figure 26) as well as on the blood vessels (Figure 27), glial cells (Figure 28) and neuronal axons of the spinal cord (Figure 29) of NOD mice starting over 6-32 weeks of age. The BKB_1-R was detected using a specific antibody (sc-15045; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), an alkaline-phosphatase-labeled anti-goat IgG

secondary antibody and the Fast Red substrate (Sigma-Aldrich, Toronto, ON, Canada) was used to create a red fluorescent precipitate. Blood vessels, glial cells and neuronal axons were visualized using a primary specific antibody against von Willebrand factor protein (VWF) (sc-8068; Santa Cruz Biotechnology Inc., Santa Cruz, CA); glial fibrillary acid protein (GFAP) (IB4 556328; BD Pharmingen, Mississauga, ON, Canada) and myelin basic protein (MBP) (Myelin BP 559904; BD Pharmingen, Mississauga, ON, Canada), respectively and an Alexa-488-labeled anti-rabbit IgG secondary antibody for detection (green colour; Biogenex, San Ramon, CA). Nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI) (blue colour; Biogenex, San Ramon, CA, USA). (unpublished results).

(a) Wester Blot



(b) Real -Time PCR

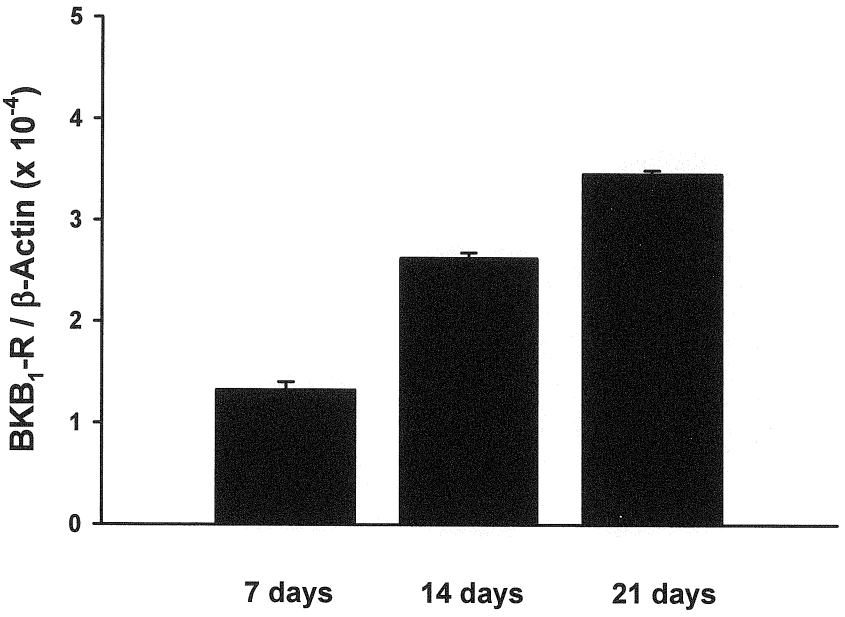


Figure 23. Evaluation of the expression of the BKB_1-R in the pancreas of control and STZ-diabetic mice by (a) Western blot and (b) real time PCR.

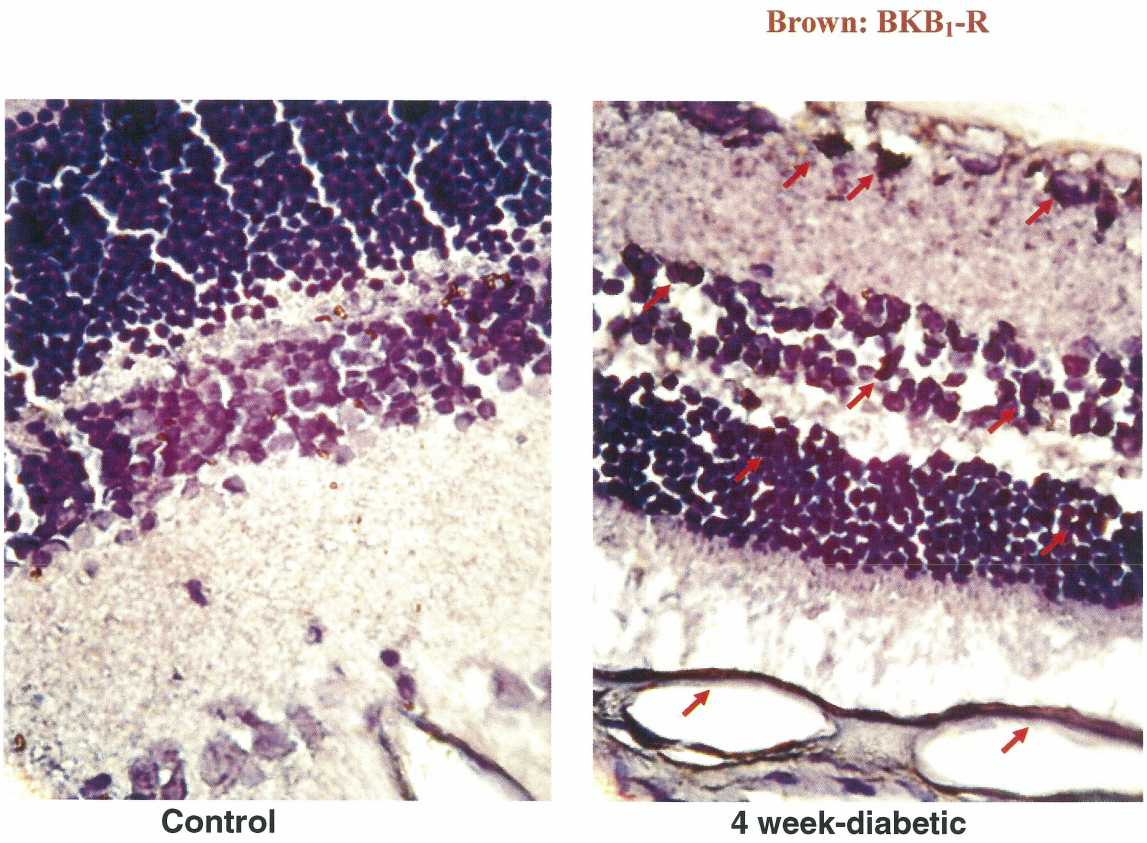


Figure 24. Immunohistochemical staining of the BKB_1-R on the retina and choroid tissues of control and STZ-diabetic Wistar rats.

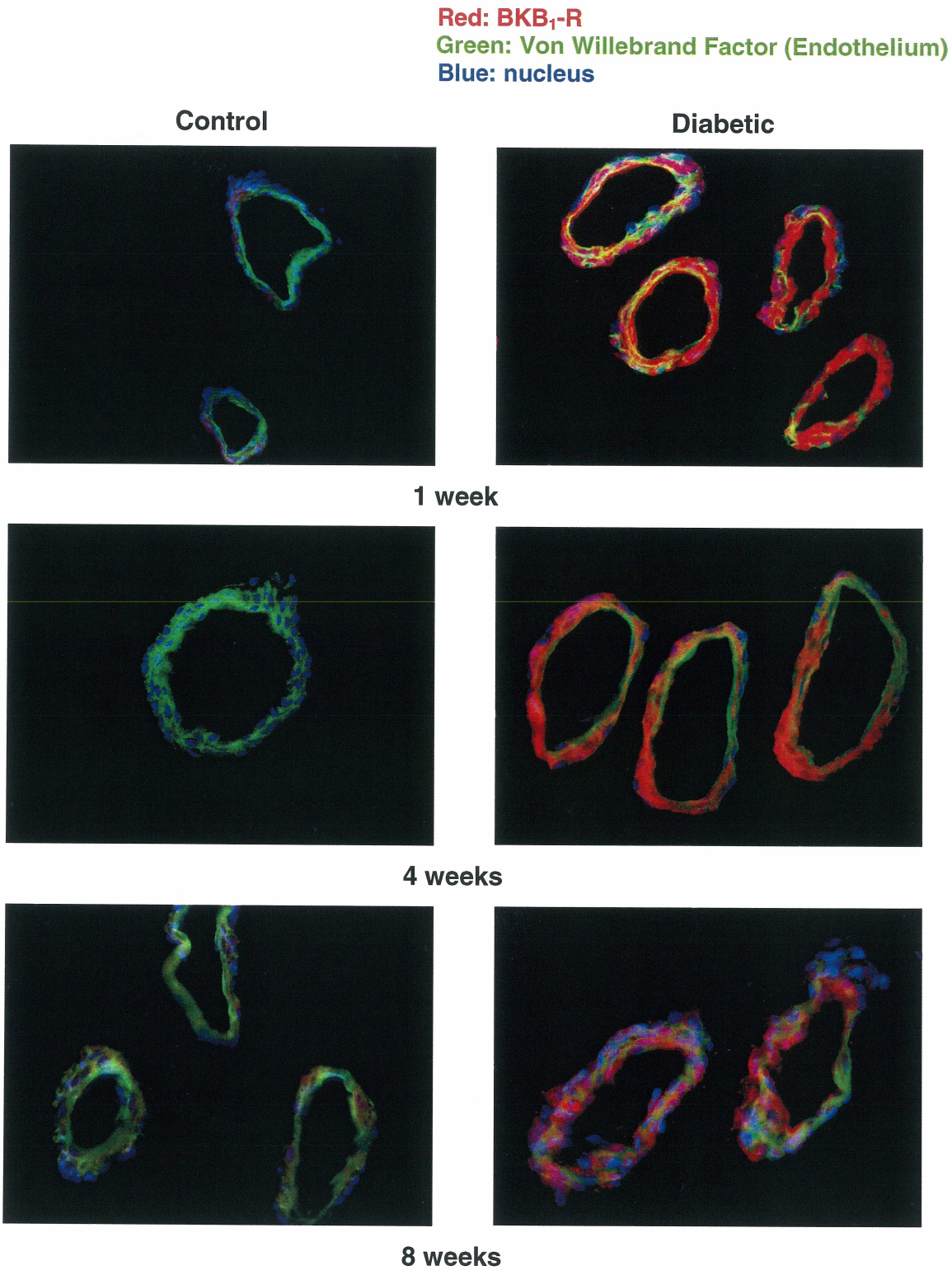


Figure 25. Immunohistochemical co-staining of the BKB_1-R and endothelium on blood vessels of the skin of control and STZ-diabetic Wistar rats.

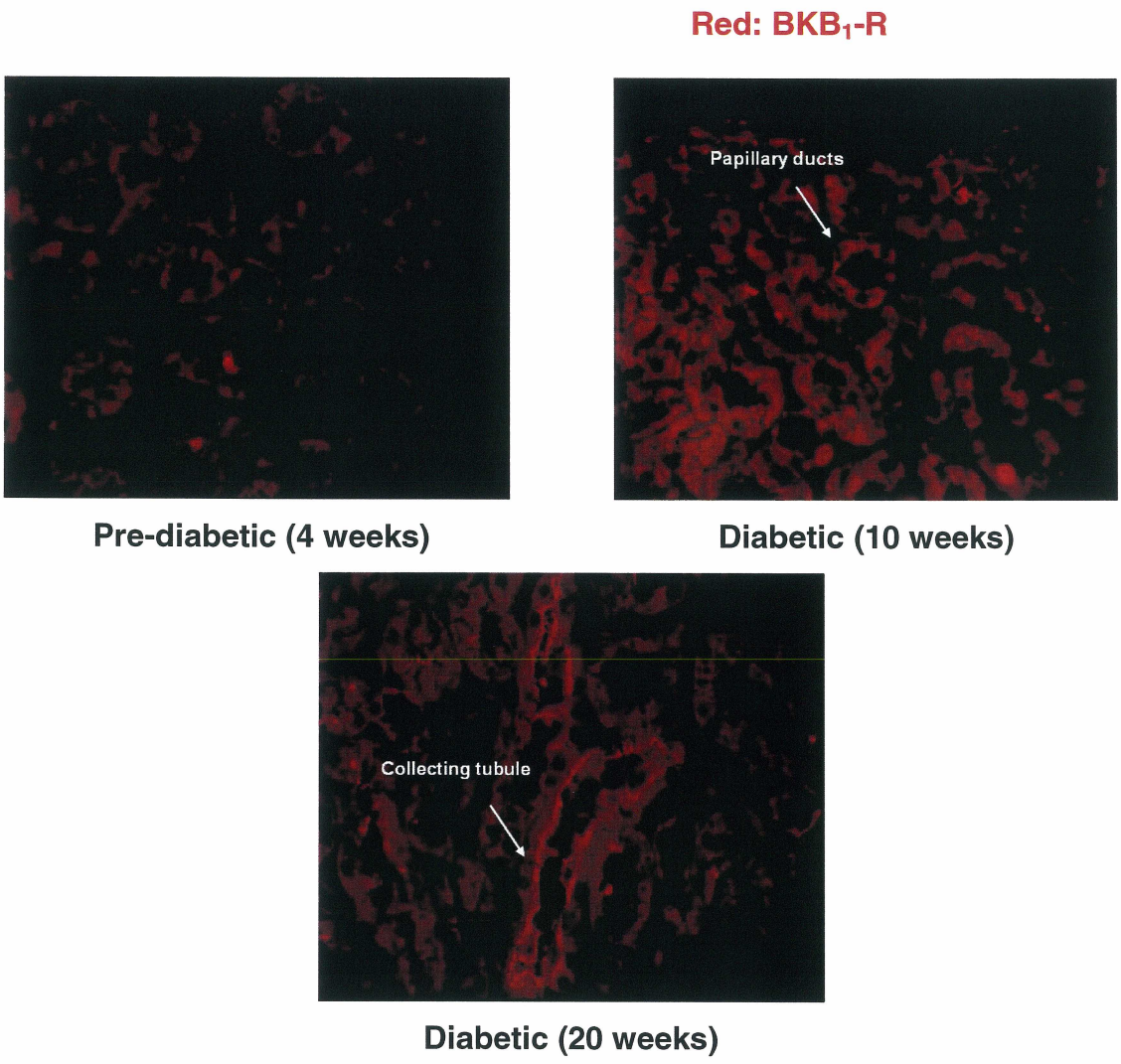


Figure 26. Immunohistochemical staining of the BKB_1-R on papillary ducts and collecting tubules of the kidney of NOD mice.

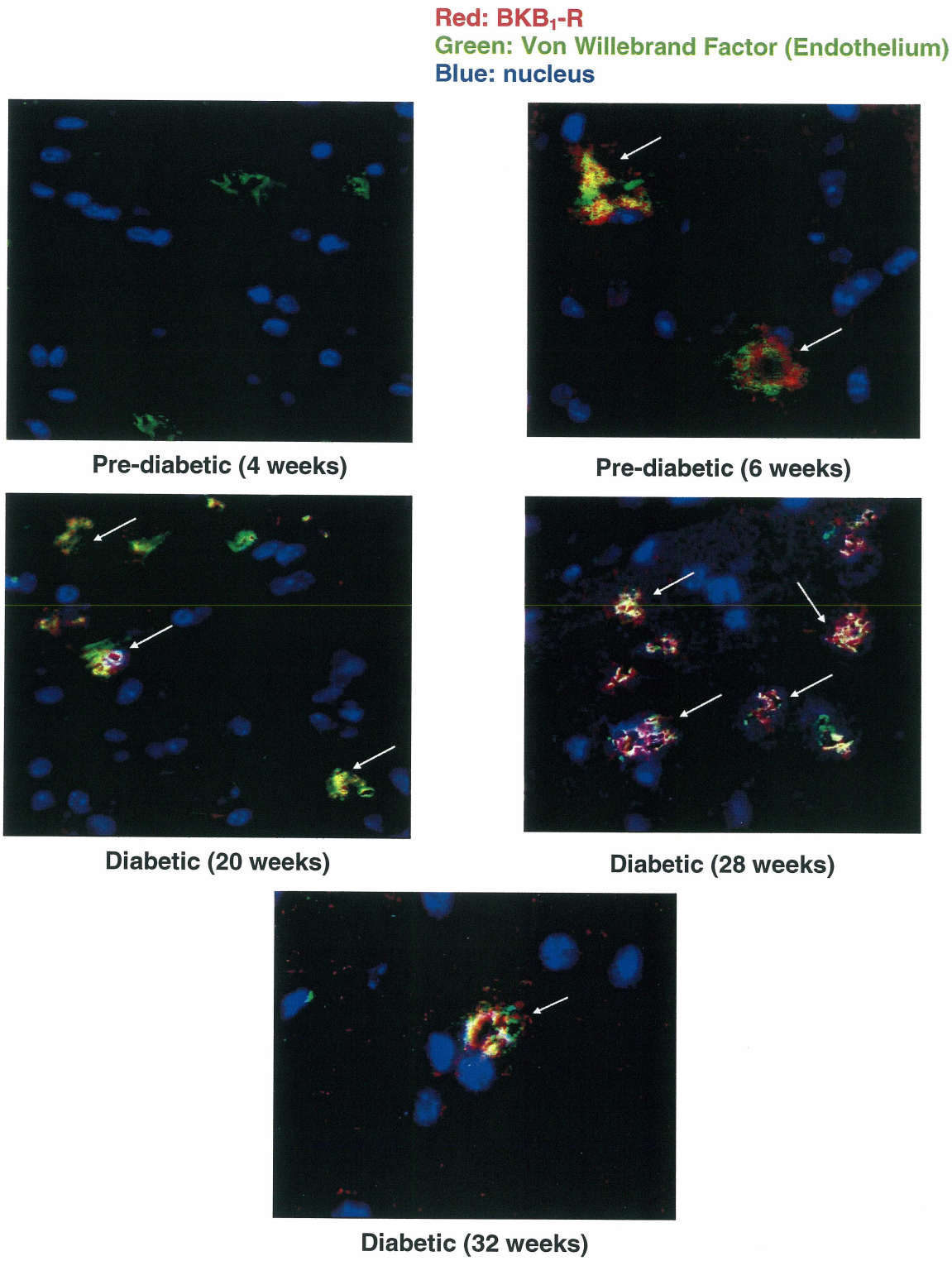


Figure 27. Immunohistochemical co-staining of the BKB_1-R and endothelium on blood vessels of the spinal cord of NOD mice.

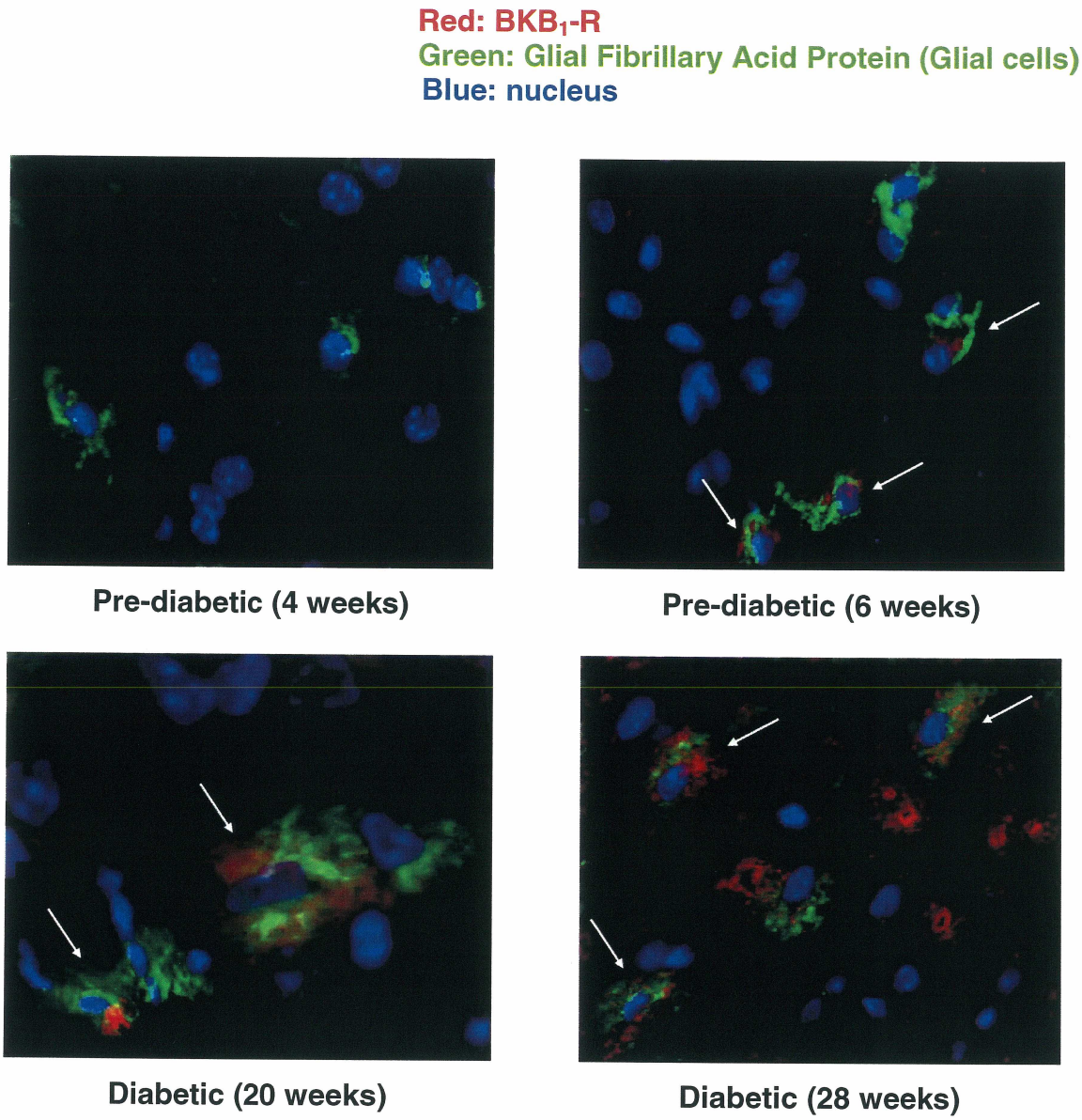


Figure 28. Immunohistochemical co-staining of the BKB_1-R and glial cells in the spinal cord of NOD mice.

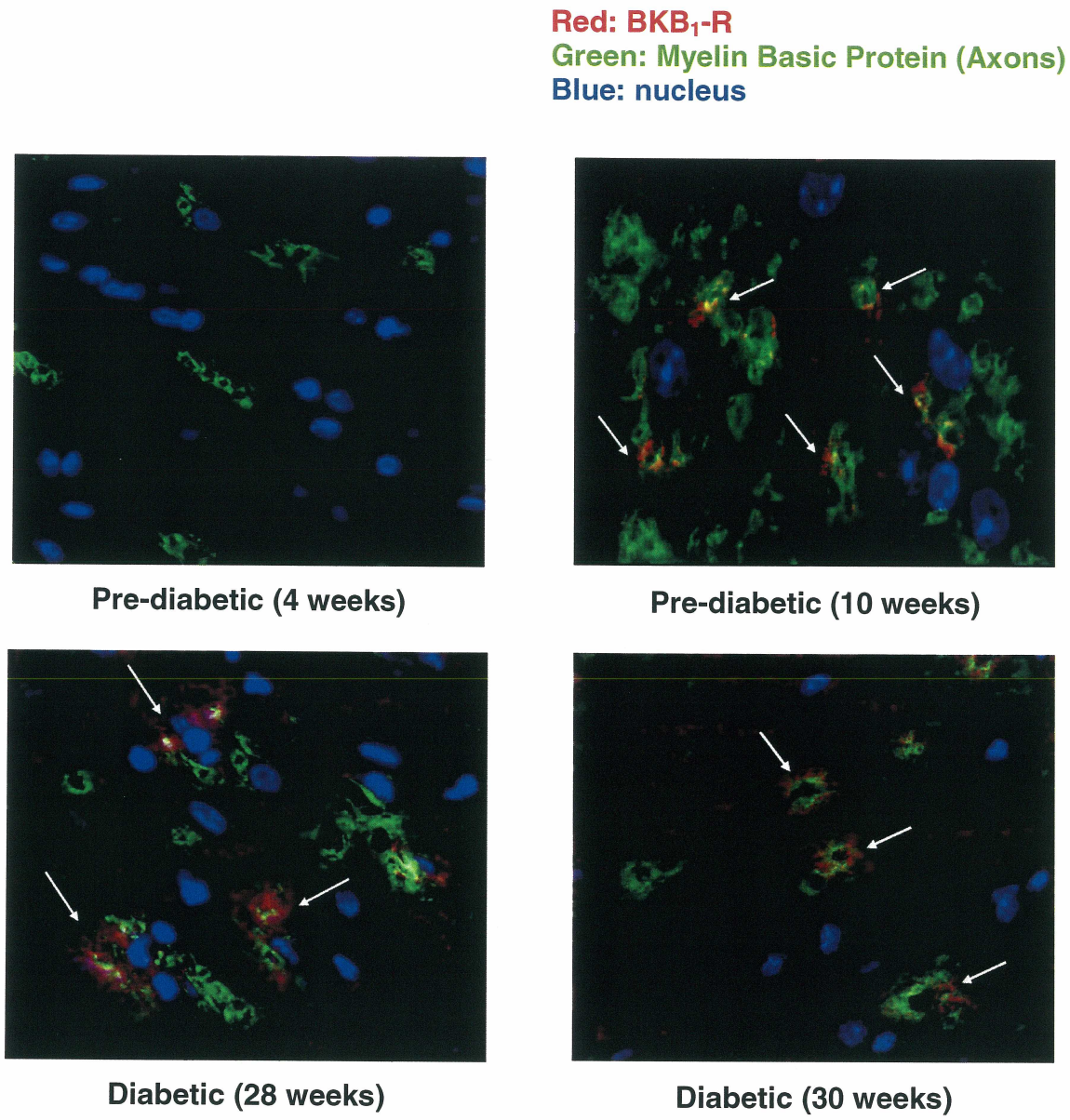


Figure 29. Immunohistochemical co-staining of the BKB_1-R and neuronal axons in the spinal cord of NOD mice.

3. Effects of insulin on hyperglucosuria and hyperalgesia in STZ-diabetic mice

3.1. Methods and results

Type 1 diabetes was induced in male CD-1 mice with a single dose of STZ (200 mg/kg, i.p.). On day 7 following STZ administration, mice started to receive a once daily s.c. dose of insulin (5 IU/kg) for 2 weeks. The urine glucose and the hot plate latency were followed over a period of 24 h after the first injection of insulin and at 1 and 2 weeks later. Our results showed that single daily treatment with insulin was able to reverse the hyperglucosuric state in STZ-diabetic mice but had no effect on hyperalgesia over the 2 weeks of treatment (Figure 30). After the first dose of insulin, the elevated urine glucose level was significantly decreased at 1.5 h, normalized over 3-6 h and increased again by 18 h post-insulin. Conversely, the mice remained hyperalgesic all the time with no change in their hot plate latency. A similar profile was observed on day 7 and day 14 of chronic insulin treatment (unpublished results).

4. Effects of R-954 on increased vascular permeability in STZ-diabetic Wistar rats

4.1. Methods and results

In a final goal, our studies aimed at evaluating the effects of the potent BKB_1-R antagonist, R-954, which significantly attenuated hyperalgesia in animal models of type 1 diabetes, on the increase in the cutaneous vascular permeability in diabetic rats, which accounts partly for peripheral neuropathy as well as the increase in retinal vascular permeability, which leads to retinopathy.

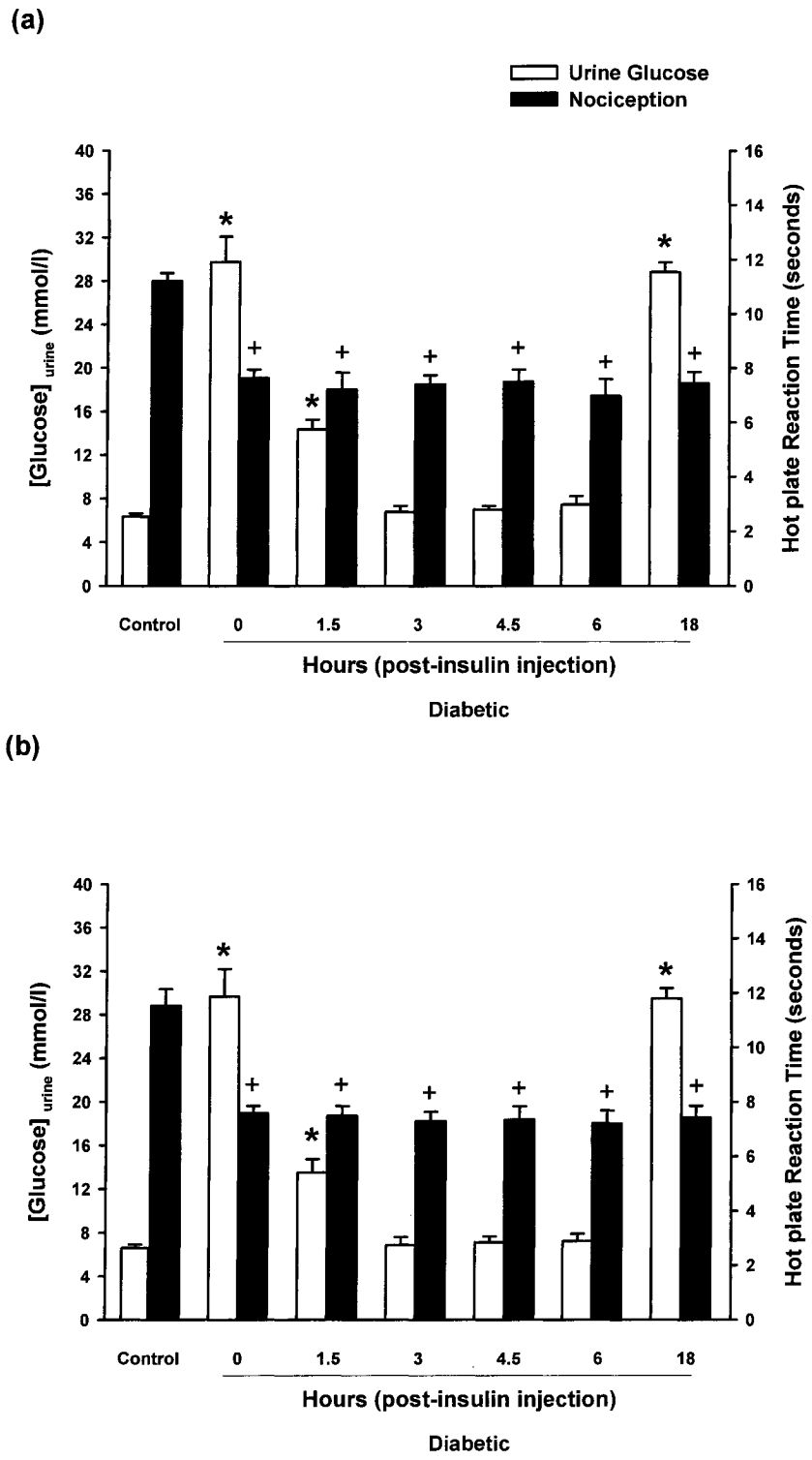


Figure 30. Effects of daily treatment with insulin on urine glucose level and hyperalgesia in STZ-diabetic mice (a) following the first dose of insulin and (b) on day 14 of insulin administration.

The capillary permeability to albumin was measured by quantifying the extravasation of albumin-bound Evans blue (EB) dye in the skin and retina of STZ-diabetic rats at 1 and 4 weeks following STZ administration.

Our results (LAWSON *et al.*, 2005b) showed a marked increase in plasma extravasation in the skin and retina of STZ-diabetic rats over 1 to 4 weeks following STZ injection. Acute treatment with the selective BKB_1-R antagonist R-954 (2 mg/kg, bolus s.c., 2 h prior to Evans blue dye) significantly reduced the elevated vascular permeability in both tissues at 1- and 4-week STZ-diabetic rats (Figure 31).

In addition, our ongoing studies have shown a significant increase in the cutaneous vascular permeability in NOD mice starting at the age of 8 weeks (prior to reaching the state of hyperglycemia) and remaining elevated up to 32 weeks of age. A similar profile has been observed with BB/Wor-DP rats in which we have reported an increase in plasma extravasation in skin compared to BB/Wor-DR rats starting also at the age of 8 weeks (before the animals become hyperglycemic) and remaining elevated up to 24 weeks of age (unpublished results).

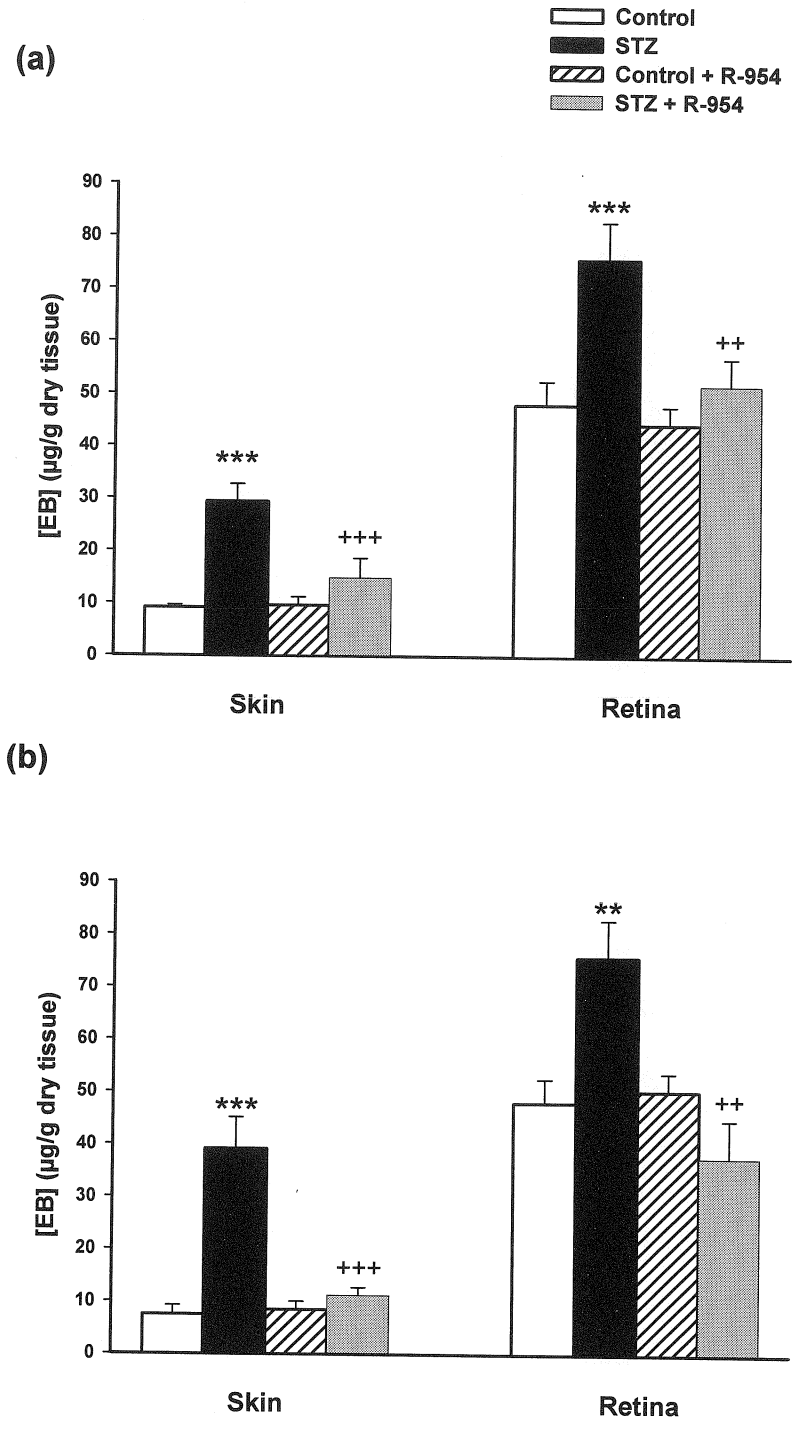


Figure 31. Effects of the selective BKB_1-R antagonist R-954 on skin and retinal vascular permeability changes in (a) 1-week STZ-diabetic and (b) 4-week STZ-diabetic rats.

DISCUSSION

1. Animal models of type 1 diabetes

The present project aimed at studying the development of hyperalgesia in rodent models of type 1 diabetes and characterizing the role of the inducible BKB_1-R in this diabetic complication through the use of selective BKB_1-R agonists and antagonists. Nociception was evaluated with two types of thermal pain tests; spinal (tail immersion and tail flick tests) and supra-spinal (hot plate and plantar stimulation) tests.

Various animal models of type 1 diabetes have been developed and used to study the etiology of diabetes and its related complications (HANNINEN *et al.*, 2003). In a part of our study, we were interested in the widely used STZ-induced model of type 1 diabetes known to ablate pancreatic β cells and to induce severe, but mostly non-lethal insulin-deficient diabetes (COURTEIX *et al.*, 1993), a wasting disease with high blood cytokine levels (LEEB-LUNDBERG *et al.*, 2005). STZ is an antibiotic extracted from *streptomyces acromogens*, which is selectively toxic for pancreatic islet β-cells. The decomposition products of STZ are known to alter the cellular membrane proteins so that they are no longer recognized as self and thus initiating an autoimmune inflammatory process associated with over-production of pro-inflammatory cytokines (Wilson and Leiter, 1990; Lukić et al., 1998) resulting in the destruction of pancreatic β-islets. In addition, the cytotoxic effects of STZ include other mechanisms such as methylation of the deoxyribonucleic acid (DNA), free radical production and NO generation. These processes are proposed to initiate DNA strand breaks resulting in the activation of the nuclear enzyme poly(adenosine diphosphate (ADP)-ribose) polymerase (PARP) as part of the DNA repair mechanism. PARP utilizes nicotinamide

adenine dinucleotide (NAD^+) as substrate to produce the ADP-ribose units required for the excision-repair process. Exhaustion of NAD^+ leads to cessation of all NAD^+-dependent cellular functions and death of the pancreatic β–cells (OKAMOTO, 1985; TANAKA *et al.*, 1995).

Our results have shown that the STZ-administered animals develop type 1 diabetes over the time. This was confirmed by evaluating the physiological markers of diabetes in rats including hyperglycemia, hypoinsulinemia, hypercholesterolemia, hypertriglyceridemia, polydipsia and polyphagia (LAWSON *et al.*, 2005a).

The STZ model reflects similar symptoms to those observed in diabetic patients with painful diabetic neuropathy, by developing tactile allodynia (CHEN *et al.*, 2002; KHAN *et al.*, 2002) and hyperalgesia to mechanical (FOX *et a*l., 1999; DOBRETSOV *et al.*, 2003; JOSEPH & LEVINE, 2003; VILLETTI *et al.*, 2003), thermal (ALEY & LEVINE, 2001; CAMERON *et al.*, 2001) and chemical stimuli (FRESHWATER *et al.*, 2002). However, this chemically-induced model of type 1 diabetes remains constantly hyperglycemic, as with poorly controlled diabetic patients and the forms of diabetes that the physicians treat today are no longer wasting diseases. Therefore, some observations based on the STZ models might have an uncertain significance for the complications of diabetes in modern-day practice (LEEB-LUNDBERG *et al.*, 2005) and consequently we used other models of type 1 diabetes in comparison.

The model of spontaneous autoimmune diabetes in non-obese diabetic (NOD) mice involves a long-term inflammatory process that closely resembles the human type 1 diabetes (TISCH & MCDEVITT, 1996). NOD mice arose from out-bred Swiss mice in Japan in 1980. Mating between brothers and sisters were used to produce a strain in which all mice develop cataract. At an early generation of inbreeding, mice without cataract but with elevated fasting

blood glucose level were noted. These were selectively bred to create a mouse model of spontaneous diabetes which results from a $CD4^+$ and $CD8^+$ T-cell-dependent autoimmune process directed against the pancreatic β-cells (SERREZE & LEITER, 1994; TISCH & MCDEVITT, 1996). The major histocompatibility complex (MHC) of the NOD mice (designated $H2^{g7}$) contributes the main component of susceptibility (similar to humans). The MHC class II I-Aβ chain shows the same diabetogenic amino acid substitution (at residue 57) associated with a high risk of development of type 1 diabetes in humans (ATKINSON & LEITER, 1999). The NOD mice develop inflammation of pancreatic islets (insulitis) at 3 weeks of age, but do not begin to develop diabetes until 10 weeks later (DELOVITCH & SINGH, 1997). There is sexual dimorphism in the incidence of diabetes in NOD mice in most of the colonies. The disease occurs earlier and more often in females, with an incidence reaching up to 70%, compared to males in which the overall incidence remains below 20% (FITZPATRICK *et al.*, 1991).

The BioBreeding/Worcester (BB/Wor) rats provide a spontaneous diabetic prone autoimmune model of type 1 diabetes, closely resembling humans (LIKE *et al.*, 1986; PARFREY *et al.*, 1989), in which the susceptibility to diabetes is linked to the major histocompatibilty complex (MHC) $RT1^u$ (COLLE *et al.*, 1981). Key features include abrupt onset of insulin-dependent, ketosis-prone diabetes between 60 and 120 days of age (NAKHOODA *et al.*, 1977) and lymphocytic insulitis (occurring before and during the acute phase of hyperglycemia) with virtually complete destruction of the pancreatic β cells (LOGOTHETOPOULOS *et al.*, 1984). This model also reflects similar symptoms to those observed in diabetic patients with neuropathy and have been used to define neuropathy-functional abnormalities (ZHANG *et al.*, 2002; STEVENS *et al.*, 2004).

2. Nociception tests

Hyperalgesia reflects an exaggerated pain response to normally noxious (painful) stimuli. It is evoked by a sensitization of peripheral C and Aδ nociceptors in addition to a central facilitation of pain transmission at the level of the dorsal horn neurons and thalamus. Conversely, allodynia is a pain due to a stimulus that does not normally provoke pain. It is evoked by sensitisation and/or reorganisation of Aβ nociceptors, loss of inhibitory controls and distortion of afferent impulse barrage (Figure 32).

Throughout our studies, we used thermal nociception tests to assess the development of hyperalgesia in animal models of type 1 diabetes. The thermal pain tests are generally classified according to (i) pain reflexes, (ii) structures involved in mediating pain response and (iii) mechanisms of pain control into spinal and supra-spinal pain tests.

The spinal pain tests (tail immersion, tail flick) produce reflexes that are relayed in the distal part of the spinal cord and persist after spinal cord trans-section (IRWIN *et al.*, 1951), indicating that the motor response does not rely on cognitive processing and requires minimum coordination. They are controlled by the descending pain inhibitory mechanism (NECKER & HELLON, 1978; BASBAUM & FIELDS, 1984) which involves descending pathways from the midbrain and the brain-stem that exert a strong inhibitory effect on the dorsal horn transmission, mostly mediated through the endogenous opioid system (RAMABADRAN *et al.*, 1989). On the other hand, the supra-spinal pain tests (hot plate and plantar stimulation) produce coordinated reflexes which involve a higher degree of cognitive processing (RAMABADRAN & BANSINATH, 1986).

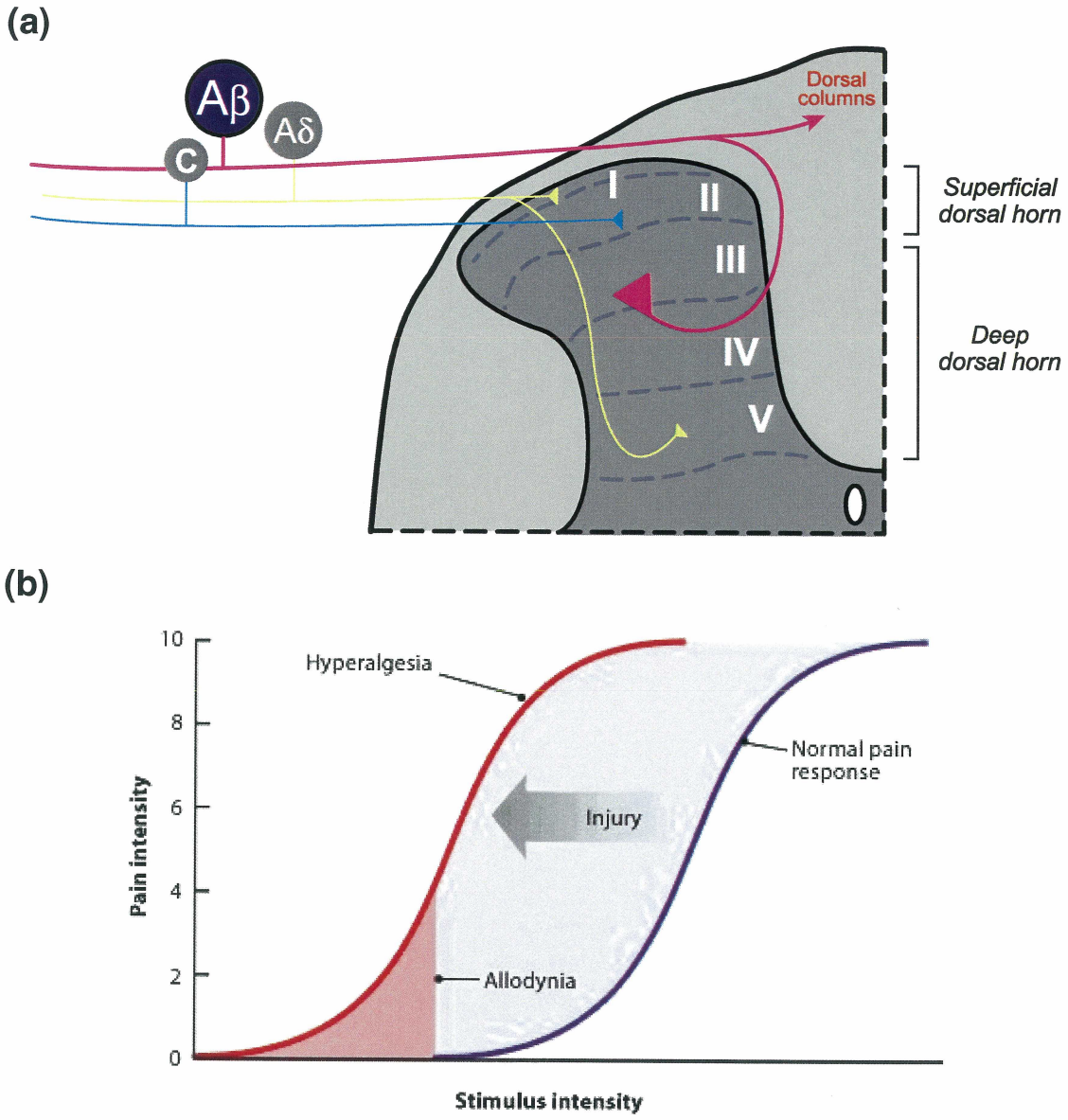


Figure 32. (a) Nociceptive (C and Aδ) and non-nociceptive (Aβ) fibres terminating in the dorsal horn layers in the spinal cord. (b) Noxious stimuli and pain response. Noxious stimuli can sensitize the nervous system response to subsequent stimuli. The normal pain response as a function of stimulus intensity is depicted by the curve at the right, where even strong stimuli are not experienced as pain. However, a traumatic injury can shift the curve to the left. Then, noxious stimuli become more painful (hyperalgesia) and typically painless stimuli are experienced as pain (allodynia).

In addition to the descending pain inhibitory mechanism, supra-spinal pain responses are also controlled by the gate control theory of pain (MELZACK & WALL, 1965; FIELDS & LEVINE, 1984) which regulates the passage of impulses from the peripheral afferent fibers to the thalamus via transmission neurons originating in the dorsal horn of the spinal cord.

The tail flick test and the plantar stimulation test have an advantage over the tail immersion and the hot plate test, respectively in that the reaction times as well as the cut-off time are determined automatically in order to minimize the experimenter error.

3. Diabetic hyperalgesia

Our results showed that the chemically (STZ)-induced diabetic mice and rats develop a marked acute thermal hyperalgesia that is stable over 4 weeks following the STZ injection. This was evaluated by spinal and supra-spinal thermal nociception tests. Treatment of mice with a daily subcutaneous dose of insulin (5 IU/kg) normalized the urine glucose level of STZ-diabetic mice but had no effect on hyperalgesia over 2 weeks.

Our results also brought the first evidence for the development of diabetic hyperalgesia in NOD mice, a model of autoimmune spontaneous type 1 diabetes. The NOD mice developed significant hyperalgesia over time (4-32 weeks of age) starting from the 8^{th} week of age with the maximum effect observed over 16-20 weeks reaching a plateau up to the 32^{nd} week. Similarly, we demonstrated that the BB/Wor-DP rats, another model of gene-prone type 1 diabetes developed a time-dependent hyperalgesia (over 4-24 weeks) starting at the age of 6 weeks and stabilizing to a maximum over the age of 16-24 weeks. Treatment of both models with insulin maintained a controlled glycemia with no significant effect on hyperalgesia.

Moreover, we showed that the hyperalgesia observed in NOD mice and BB/Wor rats did not correlate with hyperglycemia, but rather preceded the increase in the animal plasma glucose concentration. This finding supports the hypothesis that type 1 diabetic complications start to develop during the early inflammatory progression of the disease, even before establishing the hyperglycemia and/or the glucosuria-based diagnosis for diabetes. This could be due to the over-production of cytokines and the oxidative stress developing during the autoimmune response in diabetes as well as the subsequent activation of the MAP-kinase and the NF-κB pathways (COUTURE *et al.*, 2001).

Nevertheless, our results have provided clear-cut evidence that the hyperalgesia observed in wild type STZ-diabetic mice is totally absent in the BKB_1-R-KO diabetic mice. Interestingly, the BKB_1-R-KO mice (backcrossed for 10 generations to C57BL/6 genetic background) did not show any difference in their basal latencies compared to wild type mice. Also, knocking-down of the BKB_1-R did not affect the rate of STZ-induced diabetes incidence. Such important findings confirm the crucial role of the inducible BKB_1-R subtype in the development of diabetic hyperalgesia. They are also in agreement with data provided by PESQUERO *et al.*, (2000) who demonstrated that in BKB_1-R-KO mice, tissue reactions to microbial toxins, local inflammatory agents, and painful thermal and inflammatory stimulations are reduced without any apparent physiological or behavioural impairments.

It is worthy to mention that our models of type 1 diabetes (STZ-diabetic CD-1 and C57BL/6 mice; NOD mice; STZ-diabetic and BB/Wor-DP rats) showed no difference in their hyperalgesia evaluated by both spinal and supra-spinal pain tests. These observations are reassuring since the literature is somewhat confusing over the effects of diabetes

on thermal latencies which may be due in part to methodological details such as the use of different species, limbs, methods of heat application, degree of temperature and age-related diabetic status (CALCUTT, 2004).

4. Up-regulation of the BKB_1-R in diabetes

The BKB_1-R subtype is absent or of little impact under normal physiological conditions (COUTURE *et al.*, 2001), but over-expressed in pathological conditions.

Our results have demonstrated the up-regulation of BKB_1-R in the pancreas of STZ-diabetic mice as well as in the retina, choroid tissues (target for retinopathy) and the blood vessels of skin (target for neuropathy) of STZ-diabetic rats (see additional unpublished results). We have also shown for the first time the expression profile of the BKB_1-R as a function of age in NOD mice in selected tissues including the kidney and spinal cord of NOD mice. We demonstrated the expression of the BKB_1-R on the blood vessels, glial cells and neuronal axons in the spinal cord of NOD mice (see additional unpublished results)

Pharmacological evidence supports the up-regulation of the BKB_1-R subtype in STZ-diabetic animal models. It has been reported that the BKB_1-R is over-expressed in the stomach of STZ-diabetic mice since the sensitivity of the stomach fundus to the BKB_1-R endogenous agonist; DBK was substantially increased in these animals compared to control non-diabetic mice (PHENG *et al.*, 1997).

CLOUTIER & COUTURE (2000) and MAGE *et al.* (2002) reported the induction of the BKB_1-R in the kidney and spinal cord of STZ-treated mice. In addition, the up-regulation of BKB_1-R has been also shown in the lung macrophages and fibroblasts (KOYAMA *et al.*, 2000) as well as at the peripheral terminals of C-fibres and on

the endothelial cells in the lung (VIANNA *et al.*, 2003) of STZ-diabetic rats. Moreover, a recent study showed a significant increase in the level of the BKB_1-R mRNA expression in the spinal cord and brain of STZ-diabetic Wistar rats (2 and 7 days following the injection of STZ) and of its specific binding sites (2, 7 and 21 days following STZ injection) (ONGALI *et al.*, 2004; Figure 33).

The up-regulation of the BKB_1-R in type 1 diabetes is attributed to several mechanisms including the cytokines (IL-1β and TNF-α)-induced activation of the MAP- kinase and NF-κB pathways (LARRIVÉE *et al.*, 1998; NI *et al.*, 1998; SCHANSTRA *et al.*, 1998; ZHOU *et al.*, 1998; SARDI *et al.*, 1998; CAMPOS *et al.*, 1999). In addition, hyperglycemia and the resulting oxidative stress observed alongside diabetes can activate NF-κB (YERNENI *et al.*, 1999), which is known to induce the BKB_1-R (MARCEAU *et al.*, 1998). Therefore, both the over-production of cytokines and hyperglycemia could trigger the expression of the BKB_1-R through NF-κB in diabetes. Moreover, the long-term exposure of the BKB_1-R to its endogenous agonist DBK results in increased receptor expression (FAUSSNER *et al.*, 1999). Nevertheless, a synergistic interaction appears to exist between BKB_1-R ligands and IL-1β to enhance the expression of BKB_1-R (PHAGOO *et al.*, 1999). Finally, the BKB_1-R was shown to be cross up-regulated by the BKB_2-R activation (via autocrine production of cytokines and activation of NF-κB) and/or BKB_2-R sensitization (PHAGOO *et al.*, 1999).

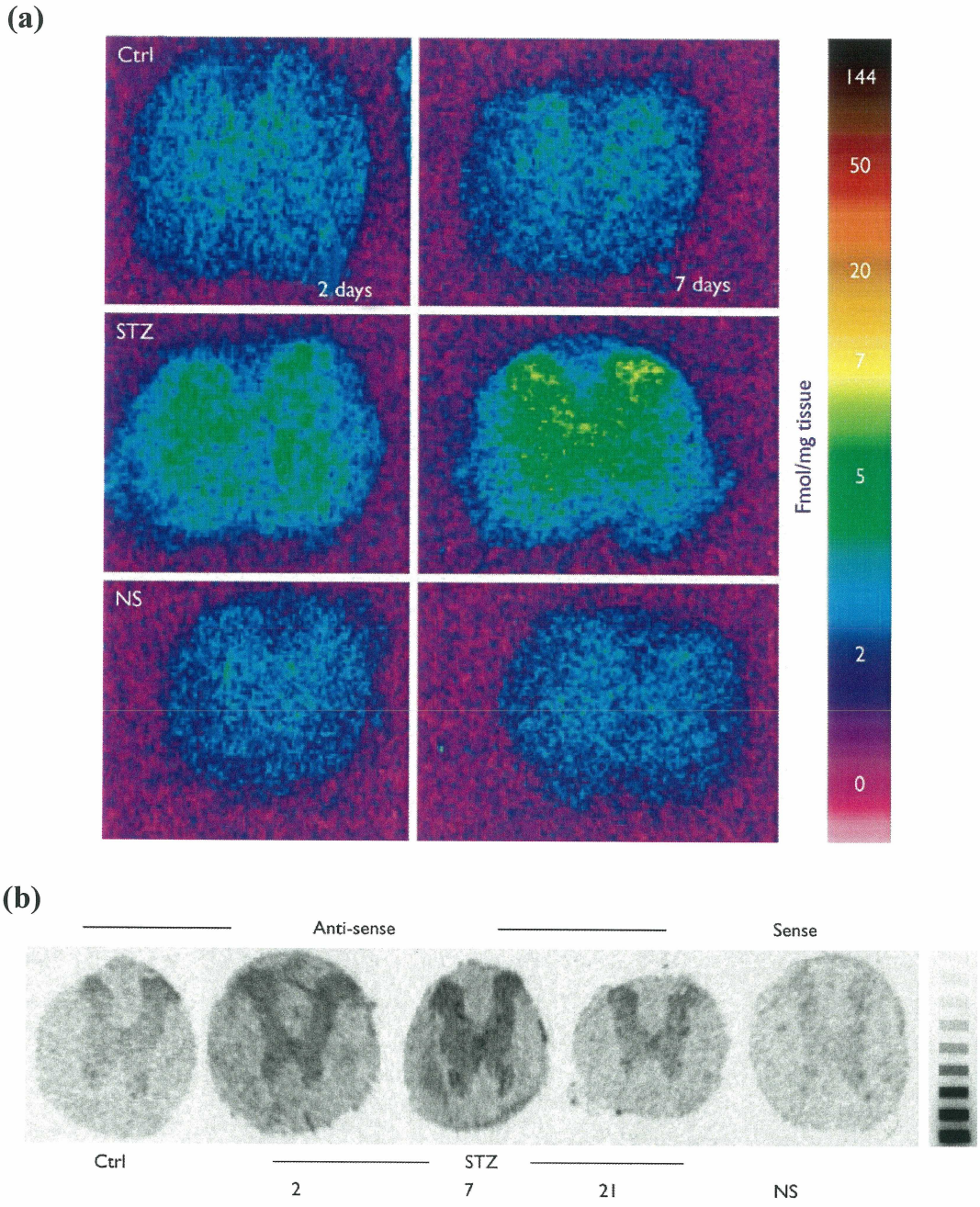


Figure 33. (a) Autoradiographic distribution and densities of the BKB_1-R binding sites in the spinal cord of control (Ctrl) and STZ-diabetic Wistar rats. The non-specific binding (NS) was obtained in the presence of 1 mM HPPdesArg^{10}Hoe140. **(b) Autoradiograms of the BKB_1-R mRNA transcripts distribution in spinal cord of control (Ctrl) and STZ-diabetic Wistar rats.** The *in situ* hybridization signal related to the expression of the BKB_1-R gene was seen all over the grey matter in diabetic rats compared to Ctrl animals.

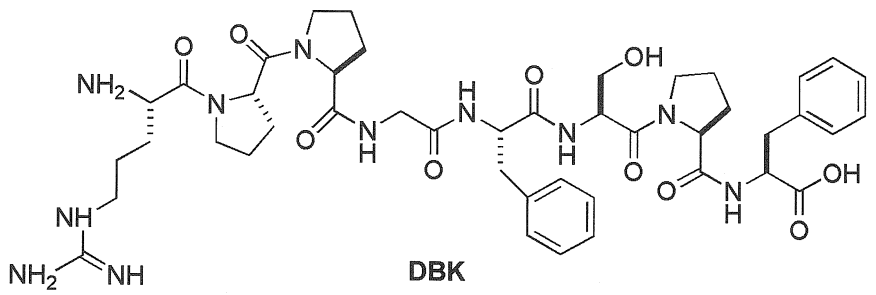
5. Peptidic BKB_1-R agonist and antagonists

In order to pharmacologically characterize the role of the inducible BKB_1-R in the development of diabetic hyperalgesia, we used the selective BKB_1-R agonist DBK as well as the selective antagonists R-715 and R-954.

The DBK (Figure 34) is an active metabolite resulting from the degradation of BK by the action of kininase I CPN with cleaves the C-terminal Arg of BK. The DBK selectively activates the BKB_1-R as its endogenous ligand. The half-life of DBK is 4- to 12-fold longer than that of BK (DÉCARIE *et al.* 1996; MARCEAU *et al.* 1998).

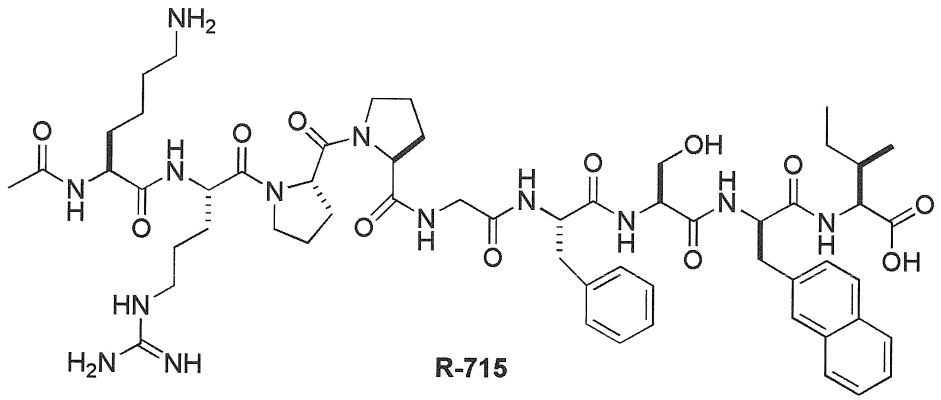
Potent and selective BKB_1-R antagonists have been developed over the past 10 years. The classical BKB_1-R antagonists such as desArg^{10}Hoe 140 (WIRTH *et al.*, 1991), [Leu^8] desArg^{9}BK and Lys-[Leu^8] desArg^{9}BK (ALLOGHO *et al.*, 1995), were found to act as partial agonists in various segments of the intestine and the urinary bladder of the mouse (ALLOGHO *et al.*, 1995) and of the rat colon epithelium (TEATHER & CUTHBERT, 1997). However, WILLE *et al.* (2001) showed that [Leu^8] desArg^{9}BK selectively inhibited the increase in vascular permeability induced by LPS in rats.

A modification of the structure of these classical BKB_1-R antagonists has led to new compounds including R-715 (Ac-Lys-[D-ßNal^7, Ile^8]desArg^{9}BK) (Figure 34) (pA2 = 7 in murine stomach; ALLOGHO *et al.*, 1995; pA2 = 8.4-8.5 in rabbit aortas and human umbilical cord; NEUGEBAUER *et al.*, 2002). The selectivity of R-715 for the BKB_1-R was established *in vitro* in murine isolated stomach, intestine and urinary bladder strips (ALLOGHO *et al.*, 1995) and in the rat isolated perfused kidney (BAGATÉ *et al.*, 1999) as well as *in vivo*, in the spontaneously hypetensive rats (EMANUELI *et al.*, 1999), in a model



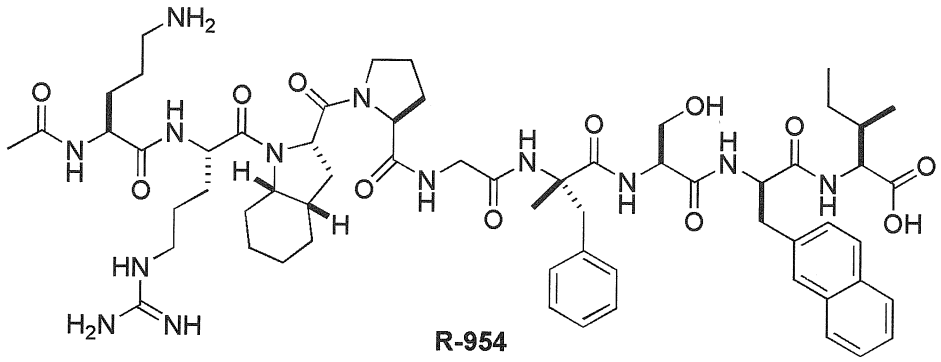
DBK

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH



R-715

Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-D-(β-Nal)-Ile-OH



R-954

Ac-Orn-Arg-Oic-Pro-Gly-α-MePhe-Ser-D-(β-Nal)-Ile-OH

Figure 34. Selective peptidic BKB_1-R agonist (DBK) and antagonists (R-715 and R-954).

of zymosan-induced inflammatory hyperalgesia in rats (BÉLICHARD *et al.*, 2000) and in normotensive rats and mice chronically treated with an ACE inhibitor (MARIN-CASTANO *et al.*, 2002).

Additional chemical modifications at the compound R-715 (α-methyl phenylalanine (α-MePhe) in position 5, L-octahydroindole-2-carboxylic acid (Oic) in position 2, and acetyl ornithine (Ac-Orn) instead of acetyl lysine (Ac-Lys) at the N-terminal) led to a more potent and long acting BKB_1-R antagonist, R-954 (Ac-Orn-[Oic^2, α-$MePhe^5$, D-βNal^7, Ile^8] desArg^9BK) (Figure 34) (pA2 = 8.4-8.6 in rabbit aortas and human umbilical cord; Ki = 2.4 nmol/l at the BKB_1-R and a Ki > 10 μmol/l at the BKB_2-R (NEUGEBAUER *et al.*, 2002). The selectivity of R-954 for the BKB_1-R was further confirmed in a Spectrum screen analysis (MPS Pharma) on 122 molecular targets (unpublished data).

6. Effect of the BKB_1-R agonist and antagonists on diabetic hyperalgesia

Our results have shown that the selective BKB_1-R agonist DBK did not affect nociceptive responses in control non-diabetic mice. In addition, DBK had no effect on pain threshold in STZ-diabetic BKB_1-R-KO mice. Conversely, DBK potentiated in a significant manner the hyperalgesia observed in STZ-diabetic and NOD mice.

On the other hand, acute and chronic treatment with the selective BKB_1-R antagonists, R-715 and R-954, significantly attenuated diabetic hyperalgesia in all tested animal models. In contrast, the selective BKB_2-R antagonist, HOE-140, did not affect hyperalgesia in diabetic animals. Moreover, the selective BKB_1-R antagonists used were able to reverse the potentiating effect of DBK on diabetic hyperalgesia.

7. Mechanisms for the BKB_1-R-mediated diabetic hyperalgesia

Once the BKB_1-R is expressed *de novo* under pathological conditions, persistent signalling occurs due to very low ligand dissociation, limited desensitization and limited receptor internalization (COUTURE *et al.*, 2001). Chronic activation of the BKB_1-R is likely to be amplified by the accumulation of DBK at the site of inflammation because of its longer half-life compared to that of BK (DÉCARIE *et al.* 1996; MARCEAU *et al.* 1998), in addition to the up-regulation of CPM which may also account for the increasing endogenous level of DBK in inflammation (SCHREMMER-DANNINGER *et al.*, 1998).

Our results have demonstrated that the stimulation of the inducible BKB_1-R subtype in diabetic animals activates several neurotransmitter systems responsible for the mediation of diabetic hyperalgesia including the SP, NO and CGRP.

We could suggest several mechanisms for the BKB_1-R-mediated diabetic hyperalgesia (Figure 35). The endogenous active metabolite DBK could directly stimulate the BKB_1-R on sensory neurones to release SP, CGRP, neurokinin A and other nociceptive neurotransmitters leading to pain transmission. Similarly, DBK can activate the BKB_1-R on cells other than the sensory neurons including macrophages, fibroblasts or endothelial cells causing the release of prostaglandins, cytokines and NO that either sensitize or activate the nociceptors (DRAY & PERKINS, 1997). Furtheremore, DBK could stimulate the BKB_1-R on sympathetic nerves resulting in the release of neuropeptides, prostanoids and sympathetic transmitters (WALKER *et al.*, 1995) that are known to sensitize nociceptive nerve terminals causing transmission of pain signals.

8. Hyperalgesia and BKB_1-R-induced increase in vascular permeability in diabetes

Our results have shown an increase in plasma extravasation in the skin and retina of STZ-diabetic rats over 1 to 4 weeks following STZ injection. Acute treatment with R-954 (2 mg/kg, bolus s.c.) significantly reduced the elevated skin and retina vascular permeability in 1- and 4-week STZ-diabetic rats (Figure 31). These findings provide the evidence that the inducible BKB_1-R subtype modulates vascular permeability in the skin and retina of type 1 diabetic rats and suggests that BKB_1-R antagonists could have a beneficial role in diabetic neuropathy and retinopathy.

In addition, ongoing studies in our laboratory show a marked increase in plasma extravasation in several tissues from NOD mice and BB/Wor-DP rats which precedes the occurrence of hyperglycemic state (unpublished data). This profile parallels with our findings in the same animal models that the hyperalgesia was significant before the plasma glucose concentration was elevated. This evidence further confirm our hypothesis that type 1 diabetic complications start to develop during the early inflammatory progression of the disease, even before establishing the hyperglycemia and/or the glucosuria-based diagnosis for diabetes.

The increase in vascular permeability induced by the BKB_1-R is believed to enable the extravasation of blood constituents that might have a sensitizing effect on pain perception. Afferent neurons (mainly C and Aδ fibres) innervating the skin can act as vasodilators neurons controlling the blood flow, the release of vasoactive peptides and the cutaneous vascular permeability. Under diabetic inflammatory conditions, plasma protein leakage induced by immune mediators including kinins, can stimulate afferent nerve fibres and enhance their excitability (ARONIN *et al.*, 1987).

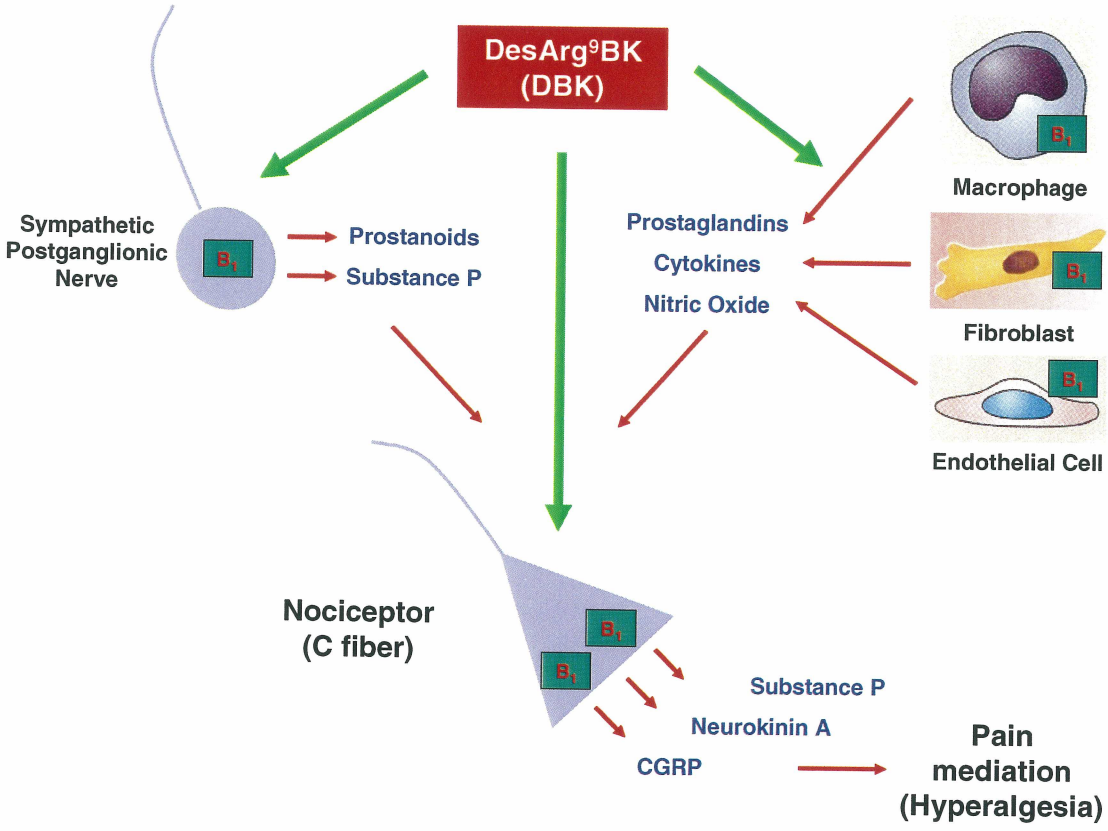


Figure 35. Mechanisms for the BKB_1-R-mediated diabetic hyperalgesia

9. The BKB_1-R and pathophysiology

The disease-specific expression of the BKB_1-R in many tissues makes it a particularly attractive molecular target for drug development. The current interest in the development of BKB_1-R antagonists is mostly related to the medical need for novel analgesic substances without the tolerance or liability for abuse that compromise the use of opiates (MARCEAU & REGOLI, 2004).

Recently, FERREIRA *et al.* (2005) confirmed the role of the BKB_1-R in chronic inflammatory pain processes. In the absence of neuropathy, the study showed no difference in the paw-withdrawal responses to thermal or mechanical stimulation between BKB_1-R knock-out mice and 129/J wild-type mice. Partial ligation of the sciatic nerve in the wild-type mouse produced a profound and long-lasting decrease in thermal and mechanical thresholds in the paw ipsilateral to nerve lesion. Threshold changed neither in the sham-operated animals nor in the paw contralateral to lesion. Ablation of the gene for the BKB_1-R resulted in a significant reduction in early stages of mechanical allodynia and thermal hyperalgesia. Furthermore, systemic treatment with the selective BKB_1-R antagonist $desArg^9[Leu^8]BK$ reduced the established mechanical allodynia observed 7–28 days after nerve lesion in wild-type mice. Partial sciatic nerve ligation induced an up-regulation in BKB_1-R-mRNA in ipsilateral paw, sciatic nerve, and spinal cord of wild-type mice. Together, BKB_1-R-activation seems to be essential to neuropathic pain development, suggesting that selective BKB_1-R antagonist might have therapeutic potential in the management of chronic pain.

We demonstrated that the BKB_1-R subtype mediates the manifestation of hyperalgesia in type 1 diabetic rodents, an effect that was blocked by the use of selective BKB_1-R antagonists. Thus, the activation of the BKB_1-R is a critical step in the production

of neuropathic pain and selective antagonism of this inducible receptor may constitute a novel and potential therapeutic approach for the treatment of painful diabetic neuropathy, since BKB_1-R is able to not only prevent the development of nociception but also reduce an established painful condition.

On the other hand, the same receptor subtype seems to be involved in a variety of diseases. Evidence suggests a pro-epileptic role for the BKB_1-R since it has been shown to be up-regulated in several brain areas (while the BKB_2-R is significantly decreased) following kindling-induced epilepsy, which is consistent with a neuronal response (potentiation of electrically evoked glutamate overflow) to a BKB_1-R agonist in hippocampal and cortical regions of rats with experimental epilepsy, an effect which is prevented by a selective BKB_1-R antagonist (BREGOLA *et al.*, 1999). The induction of the BKB_1-R in epilepsy could be related to the increased expression of IL-1β, TNF-α, NF-κB and MAP-kinase (RODI *et al.*, 2005). Therefore, BKB_1-R antagonists that penetrate the central nervous system (HESS *et al.*, 2004) could show potential benefits in epilepsy and other diseases.

In addition, STADNICKI *et al.*, (2005) demonstrated that the increase of the BKB_1-R gene expression (relative to BKB_2-R gene expression) is concomitant with an increase of BKB_1-R protein concentration in ulcerative colitis active intestine, indicating that the BKB_1-R is a major structural background for kinin function in human inflammatory bowel disease. These observations provide insight in the pathogenesis of inflammatory bowel disease and suggest that selective BKB_1-R inhibitors may have potential in a therapeutic trial.

Conversely, a protective role has been proposed for the BKB_1-R in multiple sclerosis, an inflammatory demyelinating disorder of the CNS. It is thought that an important step for the initiation of this pathology is the migration of T-lymphocytes in the CNS. High levels of BKB_1-R mRNA and protein have been found in blood T-cells of patients affected by multiple sclerosis and the addition of a BKB_1-R agonist, Sar (D-Phe^8)desArg^{9}BK, reduces their migration *in vitro*, an effect prevented by R-715, a selective B_1 receptor antagonist (PRAT *et al.*, 1999). Moreover, a decrease of BK and a significant increase in DBK blood concentration have been demonstrated in experimental allergic encephalomyelitis, a model of multiple sclerosis, suggesting an activation of carboxypeptidases involved in the transformation of BK into DBK (GERMAIN *et al.*, 1988). An up-regulation of the BKB_1-R on brain vascular endothelial and perivascular inflammatory cells has been observed in multiple sclerosis specimens (PRAT *et al.*, 2000). In keeping with this finding, experiments in human brain endothelial cells suggest that the binding of DBK to its vascular receptor contributes to an increase in the blood-brain barrier permeability to fluids and large molecules during multiple sclerosis. However, DBK may also reduce immune cell infiltration into the brain (PRAT *et al.*, 2000). Therefore, the BKB_1-R may represent a control-point for inflammation in the CNS and BKB_1-R analogues may be useful in the therapy of this disease. The critical point here is the dual effect of BKB_1-R activation: the use of an antagonist would be appropriate to block the increased permeability of the blood-brain barrier while an agonist would be more beneficial by preventing the infiltration of T-lymphocytes in the CNS. Both types of molecules need to be tested in an animal model of multiple sclerosis to address this fundamental issue.

Moreover, in a model of porcine endotoxin shock, BKB_2-R blockade attenuated LPS-induced shock mortality whereas additional BKB_1-R blockade reversed these beneficial effects. This suggests that in this animal model the BKB_1-R does not serve the same purpose as the BKB_2-R, and that the up-regulation of BKB_1-R during LPS shock may be an important mechanism of host defence (SIEBECK *et al.*, 1996).

Finally, while antagonism of the BKB_2-R in stroke, an inflammatory cardiovascular disease as arterial hypertension, reduces the infarct volume, the neurological deficit, the cerebral oedema, the blood-brain barrier disruption and the neutrophil accumulation in mice and rats submitted to a transient focal cerebral ischemia (ZAUSINGER *et al.*, 2002; DING-ZHOU *et al.*, 2003), the use of BKB_1-R antagonists have been found to be detrimental for neuronal recovery and survival, indicating that BKB_1-R activation is implicated in the late phase of stroke-induced inflammatory phenomena, i.e. those involved in tissue repair. Thus, selective BKB_1-R agonists may be of therapeutic value in this phase (RODI *et al.*, 2005).

CONCLUSIONS

Kinins are autacoid peptides and central neuromediators involved in cardiovascular regulation, inflammation and pain. Their effects are mediated by two transmembrane G-protein-coupled receptors. While the BKB_2-R is constitutive, the BKB_1-R is inducible and up-regulated by cytokines, endotoxins or during tissue injury. The BKB_2-R is believed to play an important role in the beneficial effects of ACE inhibitors used in the treatment of cardiovascular diseases, yet it is involved in the acute phase of inflammation and of somatic and visceral pain. Conversely, the BKB_1-R participates in the chronic phase of these responses and is likely to play a strategic role in diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes.

A dual function for the BKB_1-R subtype is reported in pathological conditions in which it can exert either a protective (multiple sclerosis, septic shock and stroke) or harmful (pain and inflammation) effect. Therefore, the use of selective antagonists for this receptor subtype as clinical therapeutic agents requires a rigorous evaluation of the possible potential side effects.

Our results have shown that selective antagonism of the inducible BKB_1-R subtype is effective in attenuating diabetic hyperalgesia in several animal models of type 1 diabetes including the spontaneous autoimmune models in which hyperalgesia precedes hyperglycemia. These findings suggest that antagonism of the inducible BKB_1-R subtype may constitute a novel therapeutic target against painful neuropathy in type 1 diabetic patients.

It remains to be established if type 2 diabetic models, also developing hyperalgesia, would be improved via BKB_1-R antagonism. Since selective BKB_1-R antagonists

were also reported to inhibit nociception in other non-diabetic models, the participation of the kinin system through this inducible receptor may become more relevant in pain and analgesia. We believe that many factors, through other mechanisms, may play an important pathogenic role in nociceptive sensory neuropathies including SP, CGRP, C-peptide and neurokinin, thus opening a wide range of potential targets for therapeutic intervention.

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