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Heat stress-induced B_1 receptor synthesis in the rat: an *ex vivo* study

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1 This *ex vivo* study was performed to characterize B_1 receptor induction in rats submitted to heat stress. Changes in aortic isometric tension were recorded after a 90 min *in vitro* incubation with [des-Arg^9]-bradykinin. B_1 receptor mRNA were detected in aorta and heart using RT–PCR technique.

2 Aortic rings from sham rats did not respond to [des-Arg^9]-bradykinin. In contrast, this agonist induced a concentration-dependent relaxation of aortic rings from rats submitted to lipopolysaccharide (LPS) treatment or to heat stress 24 h earlier.

3 The concentration-dependent relaxation induced by [des-Arg^9]-bradykinin on aortic rings from heat-stressed rats was abolished by [des-Arg^{10}]-HOE 140, a selective B_1 receptor antagonist.

4 In endothelium denuded aortic rings from heat-stress rats, [des-Arg^9]-bradykinin induced a concentration-dependent constriction.

5 Pretreatment of intact aortic rings from heat-stress rats with the cyclo-oxygenase inhibitor, diclofenac (1 μM) did not prevent the concentration-dependent relaxation in response to [des-Arg^9]-bradykinin. In contrast, NO synthase inhibition with N$^{\omega}$-nitro-L-arginine methyl ester (30 μM) totally prevented the vasorelaxant response.

6 B_1 receptor mRNA were not detected in aorta and heart from sham animals but were present in tissue from heat-stressed and LPS-treated rats.

7 In conclusion, our results suggest that heat stress induces a transcriptional activation of the B_1 receptor gene. The induction of B_1 receptors leads to an endothelium- and NO-dependent vasorelaxant response to [des-Arg^9]-bradykinin.

Keywords: Heat stress; endotoxin; [des-Arg^9]-bradykinin; rat aorta rings; B_1 receptor; mRNA

Introduction

Kinins exert their vascular effects through the activation of two different types of receptors responsive to [des-Arg^9]-bradykinin and bradykinin and named B_1 and B_2 receptors, respectively (Regoli & Barabé, 1980).

The existence of those distinct bradykinin receptors, previously suspected by their selectivity for different agonists, has been confirmed recently by the cloning and sequencing of two G protein-coupled receptors in different species (MacIntyre *et al*., 1993; Webb *et al*., 1994; Menke *et al*., 1994; MacNeil *et al*., 1995; Pesquero *et al*., 1996), and notably in the rat (M. Bader, personal communication). In humans, both of these seven-transmembrane domains receptors exhibit relatively low sequence homology (Leeb *et al*, 1997). The B_2 receptor is prevalent in non pathological conditions whereas the B_1 receptor activity is upregulated following trauma such as inflammation and tissue injury (Hall, 1997). B_1 receptors are often considered as amplifiers of responses from injured tissues to kinins since they mediate biological effects similar to those of B_2 receptors. The *de novo* induction of receptors *via* transcriptional activation is an uncommon mechanism for the regulation of tissue responsiveness. Most examples of receptor induction have been reported in pathological circumstances indicating that pathological states can lead to a modification in the receptor profiles (Donaldson *et al*., 1997). The molecular mechanisms involved in this induction are actually unknown.

Moreover, most isolated smooth muscle preparations responsive to [des-Arg^9]-bradykinin need a prior *in vitro* incubation (Marceau, 1995). The responsiveness can be increased when the *in vitro* incubation follows an *in vivo de novo* B_1 receptor synthesis.

We have recently shown that [des-Arg^9]-bradykinin induced a hypotensive response in rats submitted to prior acute heat stress (Lagneux & Ribuot, 1997).

In the present study, the *ex vivo* response of rat aortic rings to [des-Arg^9]-bradykinin was tested following heat stress and compared to that observed after lipopolysaccharide treatment, a known inductor of *de novo* B_1 receptor synthesis (Regoli *et al*., 1981). We have characterized the vascular response mediated by B_1 receptors and also investigated the B_1 receptor mRNA upregulation in aorta and heart from heat-stressed rats using a RT–PCR technique.

Methods

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985). The experiments were performed on male Wistar rats (Iffa credo, France) weighing 250–350 g.

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Heat stress

Heat stress was performed as previously described (Lagneux & Ribuot, 1997). The rats were anaesthetized with sodium pentobarbitone (25 mg kg^{-1}, i.p.) and placed in an environmental chamber under an infrared light. The temperature was recorded by means of a rectal probe. Heat exposure was performed in order to increase the internal temperature to 42 ± 0.5°C for 20 min. Animals were allowed to recover for 24 h. In the sham group, the rats were only anaesthetized.

Lipopolysaccharide treatment

Lipopolysaccharide (LPS, *Escherichia Coli*, serotype 0111 : B4, 10 μg kg^{-1}) was administered after anaesthesia (sodium pentobarbitone, 25 mg kg^{-1}, i.p.) *via* the penian vein. In the vehicle-treated group, an intravenous saline injection (NaCl 0.9%, 1 ml kg^{-1}) was performed under anaesthesia (sodium pentobarbitone, 25 mg kg^{-1}, i.p.). Animals were allowed to recover for 24 h.

Experimental protocol

The rats were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (50 mg kg^{-1}). The thoracic aorta was removed and immediately placed in cold (4°C) Krebs solution composition (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, Na_2HCO_3 25, Glucose 11, $CaCl_2$ 1.8, pH 7.4, carbogenated with 95% O_2, 5% CO_2. The aorta was then cleared of connective tissues and fat and cut into ring segments 3 mm in length. Some aortic rings had their endothelium removed by gently abrading the luminal surface. The rings were mounted onto a microvessel myograph (Mulvany, model 410A) consisting of two stainless steel wires (diameter: 0.9 mm) fastened to a transducer and a micrometer and placed in a 10 ml bath filled with carbogenated Krebs solution. Isometric recordings were performed by connecting the myograph to a Mac Lab recording system (Mac Lab 4S, AD Instruments).

After 10 min of stabilization, the rings were gradually stretched to 2 g passive force and allowed to recover for 90 min in presence or absence of [des-Arg^9]-bradykinin (2 μM). During the incubation period, the solution was renewed every 15 min. The rings were then contracted twice with KCl 75 mM in presence or absence of [des-Arg^9]-bradykinin (2 μM). After washing, the rings were once again contracted KCl with 60 mM. At the end of the contraction, [des-Arg^9]-bradykinin was added at increasing concentrations ranging from 2 nM to 2 μM. The response to acetylcholine (2 μM) was studied at the end of the experiment. It was assumed that an absence of relaxation in response to acetylcholine indicated complete endothelium removal, while relaxation indicated endothelium integrity.

In one group, [des-Arg^9]-bradykinin was added after a 10 min incubation with [des-Arg^{10}]HOE140 (20 μM), a selective B_1 receptor antagonist.

Study of the relaxation mechanism

In a separate group of aortic rings from heat-stressed rats, the experimental protocol described above was applied and then, after washings, rings were once again contracted with KCl 60 mM. 50 min later, diclofenac was added to the solution (1 μM). At the stabilization of the contraction, [des-Arg^9]-bradykinin was added at increasing concentrations ranging from 2 nM to 2 μM).

After washing, rings were equilibrated during 30 min. KCl 60 mM was added along with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 μM). After stabilization, an additional dose response curve to [des-Arg^9]-bradykinin (2 nM to 2 μM) was performed.

Oligonucleotide synthesis

Oligonucleotides were synthesized by Oligoexpress (France). The following primers were used for RT–PCR : B_1R-, the anti sense primer (5′GCTTCCGATCTGGTGTTTGTC3′, pos +272, +293 relative to the ATG start codon) and B_1R+, the sense primer (5′AAAGCAGGATGCAGGCAGAGAC3′, pos +608, +587). They amplified a 337 bp PCR fragment specific for the central part of the rat B_1 receptor coding sequence. For Southern blotting we used an internal probe, 5′CCCACA-TTCCTTCTACGCTCTGTTAAAGTC3′ (pos +542, +571).

In order to test the mRNA integrity, glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH) was used as external standard. We amplified a 220 bp by GAPDH-specific PCR fragment, using the following primers: GAP3, the sense primer, 5′GCTGAACGGGAAGCTCACTGGC3′ (pos +690, +730 relative to the ATG start codon) and GAP5, the antisense primer, 5′CCTGCACCACCAACTGCTTAGC3′ (pos +512, +533).

RNA isolation

Total RNA were isolated with Trizol reagent (Gibco BRL, France) according to the manufacturer's protocol from aorta and hearts removed from sham animals ($n=4$) or from rats submitted to heat stress ($n=4$) or to LPS treatment ($n=4$) 5 or 24 h earlier. They were quantified spectrophotometrically by measurement of absorbance values at 260 and 280 nm. All OD_{260}/OD_{280} ratios were between 1.6 and 1.8. Contamination of total RNA preparations by genomic DNA traces was removed using RNAse-free DNAse I (Boehringer Mannheim, France).

cDNA synthesis and polymerase chain reaction

RT–PCR were performed on a 50 μl final volume in one step assay using a system kit (Titan™, Boehringer Mannheim, France). The mixture contained: 1 μg of total RNA, 1 μl of enzyme mix, 0.2 mmol L^{-1} each of the four deoxynucleotides triphosphate (dNTPs), 0.3 μmol L^{-1} of downstream primer, 0.3 μmol L^{-1} of upstream primer and 5 mM of DTT. Samples were reverse transcribed for 30 min at 55°C followed by 30 min at 60°C. They were then amplified using the following protocol: 1 min at 94°C, 1 min at 55°C and 1 min at 68°C for 40 cycles.

When B_1 receptor mRNA was amplified, samples were submitted to direct PCR procedure (i.e. without the RT step) in order to control potential contaminating genomic DNA amplification. 1 μg of RNA were amplified in a final volume of 100 μl with 1.25 U of Taq polymerase (Boehringer Mannheim, France), 0.4 mmol L^{-1} of each B_1 primer and 0.2 mmol L^{-1} of the four dNTPs. Samples were amplified using the following protocol: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for 40 cycles. As positive control of this direct PCR, we used 1 μg of rat DNA.

Southern blotting

To validate the results, 20 μl of the PCR products were analysed by Southern blot and hybridization with the [γ-^{32}P]-

ATP end-labelled internal probe. The filters were hybridized for 2 h at 40°C. The filters were then washed for 10 min at room temperature successively in 2× SSC and 2× SSC/SDS 1% and finally for 10 min at 40°C in 0.2× SSC/SDS 0.5%. The autoradiogram was revealed after one night at −80°C.

Drugs

The following drugs were used: sodium pentobarbitone (Sanofi santé, France), [des-Arg^9]-bradykinin (Bachem, France), [des-Arg^{10}]HOE140 (RBI, U.S.A.), acetylcholine, lipopolysaccharide, diclofenac, N$^\omega$-nitro-L-arginine methyl ester (Sigma, France). All drugs were dissolved in distilled water except lipopolysaccharide dissolved in saline (NaCl 0.9%).

Statistics

All values are given as mean±s.e.mean. The differences between the different groups were analysed by a two-way ANOVA. Difference were considered significant at the $P<0.05$ level.

Results

B_1 receptor-mediated relaxant responses

Since responses to [des-Arg^9]-bradykinin of aortic rings from sham heat-stressed rats and from vehicle-treated rats were similar, these animals were pooled in the sham group.

The optimal resting tension was statistically the same in aortic rings from sham rats, and from heat-stressed and LPS-treated rats.

If the aortic rings were not first incubated with [des-Arg^9]-bradykinin, they did not respond to this agonist (data not shown). After a 90 min incubation period, [des-Arg^9]-bradykinin induced no response in aortic rings from sham animals (Figure 1). In contrast, the B_1 receptor agonist caused a concentration-dependent relaxation of aortic rings from animals submitted to prior LPS injection or to heat stress. The selective B_1 receptor antagonist, [des-Arg^{10}]-HOE140 abolished the [des-Arg^9]-bradykinin-induced relaxation without altering acetylcholine-induced relaxation (−46.5±5.3% and −44.5±5.0% in absence and presence of the antagonist, respectively).

Endothelium- and NO-dependency of relaxation to [des-Arg^9]-bradykinin

In heat-stressed rats, removal of the endothelium abolished the relaxation in response to [des-Arg^9]-bradykinin and caused a concentration-dependent relaxation of the aortic rings (Figure 2). Pretreatment with an inhibitor of cyclo-oxygenase, diclofenac, did not modify the response to [des-Arg^9]-

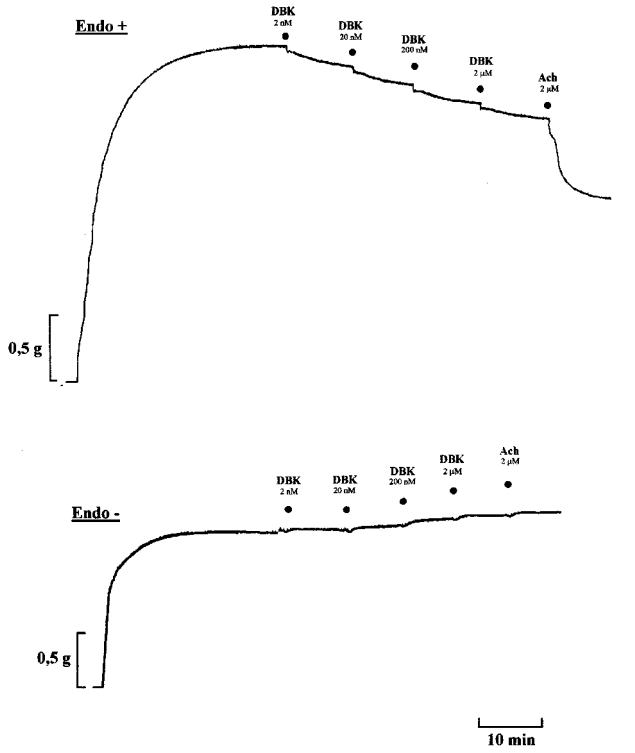

Figure 2 Typical tracings of a dose-response curve to [des-Arg^9]-bradykinin (DBK) of an endothelium intact aortic ring (upper panel, Endo+, eight rings with similar results) and of an endothelium-denuded preparation (lower panel, Endo−, eight rings with similar results) from animals submitted to heat stress 24 before the sacrifice. [des-Arg^9]-bradykinin was added after stabilization of a 60 mM potassium chloride-induced constriction. At the end of the experiment, the response to acetylcholine (ACh) was assessed. The final agonist concentrations are indicated. Time (in min) and tension (in g) calibrations are illustrated by the horizontal and vertical bars.

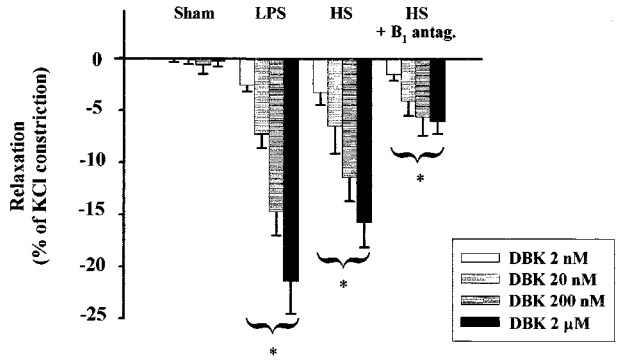

Figure 1 Concentration-dependent relaxation to [des-Arg^9]-bradykinin (DBK) of endothelium intact aortic rings from sham rats (Sham) or rats submitted, 24 h earlier, to lipopolysaccharide treatment (LPS) or heat stress (HS), without or with [des-Arg^{10}]-HOE140, a selective B_1 receptor antagonist (HS+antagonist B_1) added to the organ bath (n=eight rings for each group). *$P<0.05$ vs sham.

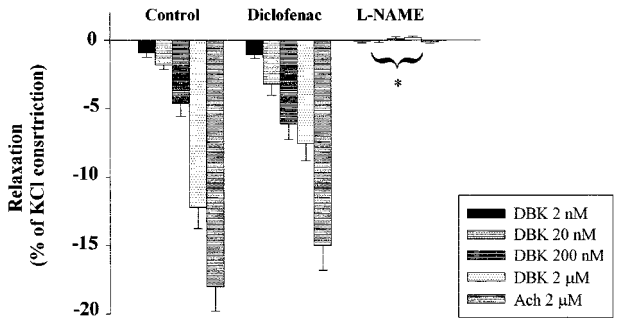

Figure 3 Responses to [des-Arg^9]-bradykinin (DBK) and to acetylcholine (ACh) in endothelium intact aortic rings from animals submitted to heat stress 24 h earlier (control), after incubation with diclofenac (1 µM) and after incubation with N$^\omega$-nitro-L-arginine methyl ester (L-NAME, 30 µM) (n=six rings for each group). *$P<0.05$ vs control.

bradykinin of aortic rings from heat-stressed animals. On the other hand, NO synthase inhibition with L-NAME totally abolished the relaxation response (Figure 3). L-NAME also abolished the acetylcholine-induced relaxation.

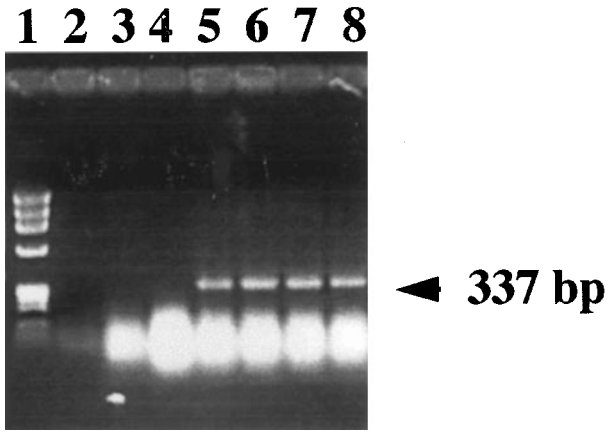

Figure 4 Tissular B_1 receptor mRNA-specific RT–PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. Samples are: The molecular weight $\varphi \times 174/HaeIII$ cut (1), no RNA (2) and RT–PCR products from sham rat aorta (3) and heart (4) LPS-treated rat aorta (5) and heart (6), heat-stressed rat aorta (7) and heart (8). The expected B_1 receptor mRNA-specific PCR fragment size was 337 bp.

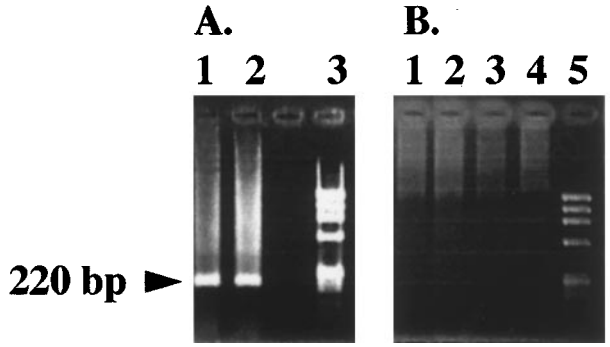

Figure 5 (A) GAPDH mRNA-specific RT–PCR were run on a 1.5% agarose gel and stained with ethidium bromide. Samples are products from sham rat aorta (1) and heart (2) and the molecular weight $\varphi \times 174/HaeIII$ cut (3). The expected GAPDH mRNA-specific PCR fragment size was 220 bp. (B) B_1 receptor mRNA-specific direct PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. Samples are: LPS treated rat aorta (1) and heart (2), heat stressed rat aorta (3) and heart (4) and the molecular weight $\varphi \times 174$ (5).

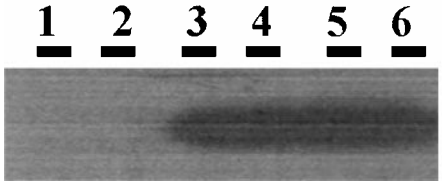

Figure 6 Southern blots: B_1 receptor-specific PCR fragment are hybridized with a ^{32}P radiolabelled probe. Samples are sham rat aorta (1) and heart (2), LPS-treated rat aorta (3) and heart (4), heat stressed rat aorta (5) and heart (6).

B_1 receptor mRNA detection

B_1 receptor mRNA-specific PCR fragment was not amplified from aorta and heart of sham animals (Figure 4) while GAPDH could be detected in these tissues (Figure 5).

B_1 receptor mRNA-specific PCR fragment was amplified from aorta and heart of rats submitted to either LPS or to heat stress treatment 5 h before the sacrifice (Figure 4). These fragments had the expected molecular size of 337 bp. B_1 receptor specific direct PCR were negative (Figure 5). B_1 receptor mRNA-specificity was confirmed by Southern blot (Figure 6).

24 h after LPS or heat stress, B_1 receptor specific PCR fragment could no longer be amplified (data not shown).

Discussion

This study provides the first demonstration that heat stress induces B_1 receptor gene transcription in the rat.

B_1 receptors are characterized by their upregulation following pathophysiological conditions. In this study, the sensitivity of the rat vascular system to [des-Arg^9]-bradykinin was increased by heat stress. We have recently shown an *in vivo* hypotensive response to this peptide in the rat following heat stress. This was attributed to a *de novo* formation of B_1 receptors which is time-dependent since the hypotensive response is effective 6 h after heat stress. This is in agreement with the observation that lipopolysaccharide must be injected at least 5 h before the experimental protocol (Régoli *et al.*, 1978).

In order to assess whether the hypotensive response to [des-Arg^9]-bradykinin observed *in vivo* was due to a peripheral vasorelaxation, we observed the *in vitro* response of aortic rings from rats submitted to heat stress 24 h earlier and compared this response with that obtained 24 h following endotoxic stress.

Most smooth muscle preparations responsive to [des-Arg^9]-bradykinin react only after an *in vitro* incubation (Marceau, 1995). In our study, a 90 min incubation period revealed the sensitivity of B_1 receptors. In these conditions, [des-Arg^9]-bradykinin induced no response in aortic rings from sham animals while it induced a concentration-dependent relaxation in rings from heat stressed animals. The same results were obtained with aortic rings from lipopolysaccharide-pretreated animals. The abolition of the relaxant response by the B_1 receptor antagonist, [des-Arg^{10}]HOE140 (Rhaleb *et al.*, 1992), is in favour of B_1 receptor implication.

We have then explored the mechanism of this B_1 receptor-mediated relaxation. While NO was proposed as the only relaxing factor in rabbit carotid arteries (Pruneau & Bélichard, 1993), an implication of prostaglandins has been described in other preparations such as rabbit mesenteric arteries (Deblois & Marceau, 1987; Ritter *et al.*, 1989) and dog mesenteric veins (Toda *et al.*, 1987). In our experimental conditions, a major involvement of NO is demonstrated by the abolition of the relaxation response by an inhibitor of NO synthase.

Moreover, removal of the endothelium reversed the response to [des-Arg^9]-bradykinin following heat stress to produce a concentration-dependent increase in tension. Similar results have been reported with acetylcholine on rat aortic rings (Luscher & Vanhoutte, 1990) and our observation suggests the presence of B_1 receptors on smooth muscle cells as well as on endothelial cells. This is in good accordance with studies that have characterized B_1 receptors on endothelial cell

cultures (Wohlfart *et al*., 1997) and smooth muscle cell cultures (Galizzi *et al*., 1994) from different species.

Finally, we have detected B_1 receptor mRNA in order to point a transcriptional activation following heat stress. Using a RT–PCR technique, we have shown that B_1 receptor mRNA, which are not present in tissues from sham rats, are upregulated after heat stress.

In conclusion, we have demonstrated that acute heat stress leads to a transcriptional activation of the B_1 receptor gene. The activation of B_1 receptors induces a vasorelaxant response from heat-stressed animals aorta rings. This response is endothelium- and NO-dependent.

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