L-Arginine Transport and Nitric Oxide Production in Kinin Receptor $B_1^{-/-}$ Endothelial Cells

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Abstract: Kinins are important vasoactive peptides, but the role of the B_1 receptor subtype in the vascular control is poorly understood. This study analyzed the nitric oxide (NO) release, L-arginine (L-Arg) uptake and the expression of the cationic amino acid transporter (CAT) -1 in endothelial cells obtained from B_1 receptor knockout ($B_1^{-/-}$) and wild type (WT) mice. NO production was assessed through a fluorescent dye in living cells stimulated with acetylcholine. L-Arg uptake was determined indirectly in the culture medium by HPLC, in the presence or absence of the CAT-1 blocker N-ethylmaleimide (NEM). CAT-1 mRNA levels and protein expression were determined by qPCR and western blot, respectively. NO release was significantly reduced in $B_1^{-/-}$ when compared to WT cells. This result was accompanied by a decreased rate in the L-Arg uptake by $B_1^{-/-}$ cells. Incubation with NEM impaired the L-Arg uptake in WT, but had no effect in $B_1^{-/-}$ cells. Protein expression and mRNA levels for CAT-1 were reduced in $B_1^{-/-}$ in comparison to WT cells. These findings suggest an important role of the endothelial B_1 receptor in the vascular control by interfering with CAT-1 expression, L-Arg uptake and NO release.

Keywords: B_1 receptor, endothelial cells, L-arginine, CAT-1, nitric oxide, knockout mice.

1. INTRODUCTION

The kallikrein-kinin system has been widely studied in view of its properties during physiological and pathophysiological processes [1,2]. Bradykinin (BK) and its metabolite des-Arg^9-BK activate two distinct G protein-coupled receptors, named B_1 and B_2. Whereas B_2 receptor is constitutively expressed and mediates many of the acute effects of kinins, the B_1-subtype is expressed at low levels in normal conditions, but rapidly induced in the course of inflammation [2]. The B_1 receptor has been widely studied at the inflammatory context; however, little is known about its specific functions in the vascular physiology. B_1 receptor knockout ($B_1^{-/-}$) mice are normotensive, but exhibit deficient hypotensive response during the first minutes after lipopolysaccharide injection [3], indicating the participation of B_1 receptors in the immediate vascular nitric oxide (NO) release. At basal conditions, $B_1^{-/-}$ mice present decreased NO levels in plasma and vascular tissue accompanied by a severe endothelial dysfunction, measured by responses to the endothelium-dependent relaxant agent acetylcholine (ACh) [4]. These studies point to an important role of the B_1-subtype in vascular NO generation, even in the absence of inflammation. However, the exact mechanism involved in this phenomenon is still unclear.

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Nitric oxide (NO) is a chemical messenger with numerous molecular targets and effects, including vasorelaxant activity after release from endothelium [5]. NO is produced from L-arginine (L-Arg) by a family of enzymes called NO Synthases (NOS), with L-citrulline as a co-product. Therefore, NO generation is largely dependent upon an adequate and continuing supply of L-Arg.

The availability of cationic amino acids in the cytoplasm is regulated via amino acid transport systems denoted by y^+, $b^{o,+}$, $B^{o,+}$ or y^+L. System y^+ is the principal system expressed in NO producing cells, playing a key role in the regulation of L-Arg supply for NOS [6]. System y^+ is inhibited by N-ethylmaleimide (NEM) [7], and the use of this sulfhydryl reagent enables to study the contribution of this system to amino acid transport in endothelial cells. At least 4 cationic amino acid transporter (CATs) isoforms have been identified in mammals, ie, CAT-1, -2A, -2B and -3, and the ubiquitous expression of CAT-1 suggests it is the principal y^+ carrier in most cells [8]. Therefore, a large number of studies regarding normal or altered vascular NO production has been focused on L-Arg uptake and its relationship with its preferential transporter CAT-1 [9-13].

The objective of the present study was to investigate the influence of B_1 receptors in the NO production at the cellular level. For this, endothelial cells obtained from $B_1^{-/-}$ mice were isolated and cultured. NO release was determined in living cells through a specific fluorescent dye, in the pres-

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ence of L-Arg supplementation. Rates of L-Arg uptake were measured by HPLC, and correlated with the CAT-1 mRNA levels and protein expression, analyzed by qPCR and western blot, respectively.

2. MATERIALS AND METHODS

2.1. Animals

C57Bl/6 wild type (WT) and $B_1^{-/-}$ male mice, aged 10-14 weeks, were obtained from the breeding stock of Centro de Desenvolvimento de Modelos Experimentais para a Biologia e Medicina (CEDEME - UNIFESP). The procedures were approved and performed in accordance with the guidelines of the Ethics Committee of the UNIFESP (protocol number 004/12HE), conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication N°. 85-23, revised 1996).

2.2. Endothelial Cells Isolation and Culture

Cell cultures were established as described by Chen *et al* [14]. Animals were euthanized by cervical dislocation, lungs were carefully removed, washed with phosphate buffered saline (PBS), cut into 1 x 1 x 1 mm pieces and placed in 6 well (35 mm) dishes. The pulmonary explants were recovered with Dubelcco's modified eagle's medium (DMEM) low glucose (Gibco / Life Tech Corp USA) supplemented with fetal bovine serum (FBS, 20%) and gentamicin (40 mg/L), pH 7.4, and placed into a CO_2 incubator (Sheldon Mfg. Inc. USA) (37 ° C). Tissues were removed after 60 h and medium was changed every 2 to 3 days. Cells grew to confluence and propagated in 1:4 ratio using 0.25 % trypsin-EDTA (Gibco / Life Tech Corp USA). The primary cultures from both WT and $B_1^{-/-}$ were characterized by *Ulex europaeus* lectin agglutinin I (UEA-1) binding and through immunofluorescence to von Willebrand Factor. Cells were subcultured as previously described by Loiola *et al* [15].

2.3. NO Production

NO production was assessed using 4,5 diaminofluorescein diacetate (DAF-2 DA, Alexis – Enzo Life Sciences Inc. USA), a fluorescent cell permeable dye for NO [16]. Cells were seeded in 35 mm dishes and semi-confluent cultures were incubated with Hank's balanced salt solution containing DAF-2DA (10 μmol/L), during 30 min, at 37°C. Experiments (n=4 for each group) were performed in the presence of L-Arg supplementation (1 mmol/L), added to the Hank´s solution. Cells were stimulated with ACh (1 mmol/L) and observed in a confocal microscope (Axiovert 100M – Carl Zeiss, DE) using appropriate filter (Ex.: 488, Em.: 515). Significant auto-fluorescence was discarded by experiments performed in the absence of DAF-2DA.

In preliminary experiments, effects of ACh on NO production were analyzed during 5 minutes, with 15 seconds intervals between each image capture. Maximal NO release occurred at 270 seconds after stimulation; therefore, this point was chosen to be analyzed and compared between groups. In a separate group of WT cells, the functional presence of B_1 receptors was tested by stimulation with the B_1 agonist des-Arg^9-BK (1 mmol/L). Fluorescence in response to NO production was quantified by optic densitometry using the AxioVision 4.8. Digital Images Analysis Software (Carl Zeiss, DE), and results were expressed as arbitrary units (a.u.).

2.4. L-Arginine Uptake Assay by High-Performance Liquid Chromatography (HPLC)

WT and $B_1^{-/-}$ cells (n= 4 for each group) were seeded in 35 mm dishes and semi-confluent cultures were incubated with DMEM in the presence and absence of NEM (1mmol/L), to induce CAT-1 blockade. After 15 h, medium was collected and analyzed by HPLC, as previously described [17]. The HPLC system used is comprised of: PDA detector, Pump 600, automatic injector Autosampler all Accela (Thermo Scientific, USA), and a Phenosphere 5 μm C18, 80 A° column (Phenomenex, USA). Samples were derivatized in a 1:4 ratio with *o*-Phthalaldehyde (Sigma-Aldrich, USA) and L-Arg was separated and quantified using reverse-phase HPLC coupled a with photo diode array detector (Accela Liquid Chromatography System - Thermo Fisher Scientific Inc., USA). Chromatographic conditions were optimized for L-Arg separation as follow: phase A, 90% acetate buffer solution (10 mmol/L, pH 5), 9.5% methanol (Merck, USA) and 0.5% tetrahydrofuran (Sigma-Aldrich, USA); and phase B, 100% methanol. The flow rate was 1 mL/min and the wave length monitored was 340 nm. Retention time was used to identify peaks of interest, determined with a peptide standard. L-Arg uptake was calculated by comparing L-Arg concentration in the medium stock (84 mg/L) and after 15 h of incubation. Values were normalized by total protein level and expressed as μg of L-Arg / mg of total protein.

2.5. Analysis of mRNA Levels for CAT-1 by qPCR

RNA was extracted from cultured cells (n=4-5) using TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. RNA was dissolved in RNase-free water. Total RNA was then purified with Qiagen RNeasy MiniKit (Qiagen USA) and submitted to DNAse treatment. RNA quantity and quality was assessed by spectrophotometry using NanoDrop v3.3.0 (NanoDrop Technologies Inc. DE) and capillary electrophoresis using Bioanalyzer 2100 (Agilent Technologies Inc. USA), respectively. A commercially available Assay-on-Demand kit (Applied Biosystems USA) was used to assess CAT-1 (Mm1219060_m1) and TBP (Mm00446971_m1) gene expression. TBP was used as normalizing gene and WT was used as the calibrator group. All qPCR reactions were performed on an ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems USA) using the fluorescent Taqman methodology (TaqMan One Step RT-PCR Master Mix Reagents, Applied Biosystems USA). Four hundred ng of total RNA were used for each qPCR reaction in a total volume of 25 μL according to the manufacturer's protocol. The thermal cycling conditions were as follows: 30s at 48°C, 10 min at 95°C, and 40 cycles of 15s denaturation at 95°C and 60 s annealing at 60°C. Cq values were used as end point defined as the qPCR cycle number in which the fluorescence generated by the amplification crosses the threshold. Three replicate reactions per sample were run to ensure statistical significance. For data analysis,

we used the comparative CT method (also known as the $2^{-\Delta\Delta ct}$ method) [18] to calculate relative quantities (RQ) of gene expression among the samples. DataAssist™, software developed for quick analysis of TaqMan® real-time PCR (Applied Biosystems USA), was used to confirm the data calculations performed in Microsoft® Excel. Whenever Cq values exceeded 36 amplification rounds (Cq), quantities were considered to be undetectable or very low and measurements were taken as invalid.

2.6. CAT-1 Protein Quantification by Western-Blotting

Semi-confluent cultures (75 cm^2) (n=3-4) were collected in cold PBS and centrifuged at 1500 rpm for 5 min at 4°C. The cell pellet was suspended in lysis buffer (Tris-HCl 50 mmol/L pH 7.4, NaCl 100mmol/L and NP40 0.5%) beyond protease/phosphatase inhibitor (Halt TM Protease and Phosphatase – Thermo Scientific USA). Protein content was determined by the method of Bradford. Equal amounts of protein (30 µg) were analyzed on a 10% sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE). The proteins in gel were transferred to nitrocellulose membranes (0.45 µm) and blocked overnight with 5% (w/v) non-fat dry milk diluted in TTBS (Tris base 0.2 mmol/L, NaCl 1.4 mmol/L and Tween 20 0,1%). Membranes were incubated with polyclonal rabbit anti-mouse CAT-1 (ab37588 Abcam - UK) (1:1500) for 90 min at room temperature. Blots were washed in TTBS (3 x 5 min) and incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Abcam - UK) (1:2000), for 90 min at room temperature. CAT-1 was detected by chemiluminescence (GeneGnome System – Syngene, UK) and quantified by densitometry (Gene Tools Software, UK). Internal controls were performed by expression of anti-β actin.

Statistical Analysis

Data were subjected to Student's t-test, and *P* values <.05 were considered to be statistically significant. Each step of experiments was performed in duplicates, using cultured endothelial cells obtained from 4-5 WT and $B_1^{-/-}$ mice. Results are presented as the means ± SEM.

3. RESULTS

3.1. NO Production in Endothelial Cells from WT and $B_1^{-/-}$

By using the fluorescent dye DAF-2DA, we investigated the endothelial production of NO, comparing WT and $B_1^{-/-}$ cells. At basal conditions, no significant differences were observed between groups (not shown). When cells were stimulated with ACh, a rapid and consistent increase in the fluorescence intensity was detected, reaching maximal values at 270 seconds of stimulation. A representative image obtained from WT and $B_1^{-/-}$ cells is showed in Figure **1**.

In the $B_1^{-/-}$ cells, ACh stimulation promoted a slight, but detectable enhancement in NO release; however, response obtained from $B_1^{-/-}$ corresponded to 50% of the values obtained from WT cells. Results are demonstrated in Figure **2**.

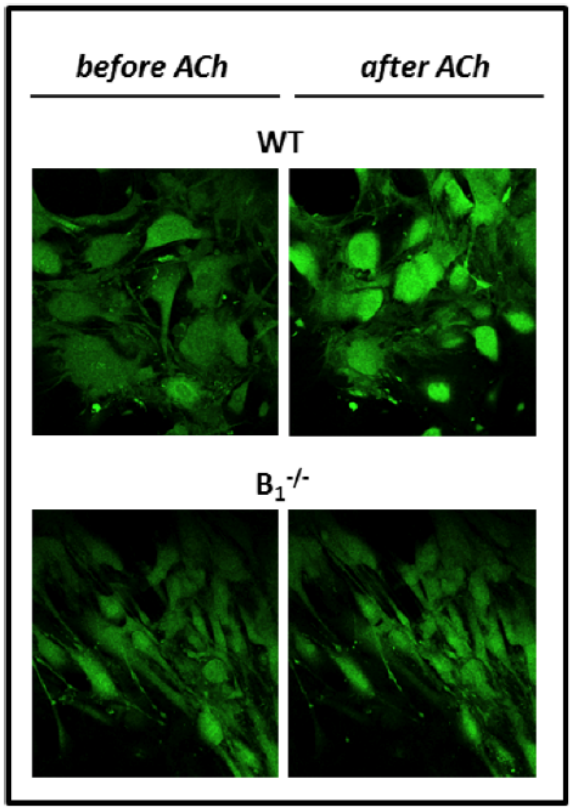

Figure 1. Representative images of fluorescence emitted by WT and $B_1^{-/-}$ endothelial cells pre-incubated with DAF-2DA, before and after 270 seconds of ACh (1 mmol/L) stimulation.

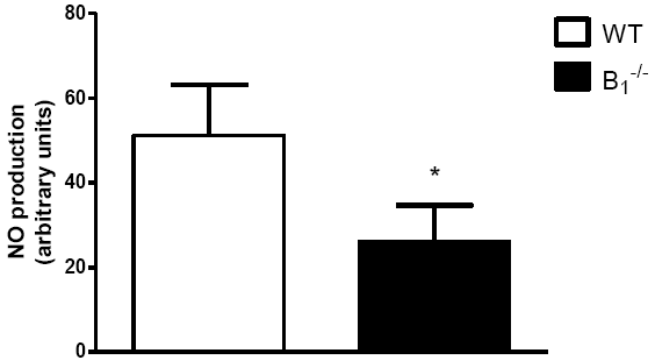

Figure 2. NO production measured by fluorescence intensity in WT and $B_1^{-/-}$ cultured cells, after ACh (1 mmol/L) stimulation, in the presence of L-Arg (1 mmol/L). Data are mean ± sem; n = 4; **P* <.05 *vs* WT.

In order to test the functional presence of B_1 receptors in WT cultured cells, images were compared before and 270 seconds after des-Arg^9-BK stimulation. In these experiments, increase in the fluorescence was slightly less in comparison to that observed for ACh, but no statistical differences were detected: 59 ± 6 for des-Arg^9-BK (a.u., n=3) *vs* 70 ± 3 for ACh (a.u., n=4).

3.2. L-arginine Uptake

Endothelial L-Arg uptake was quantified in cultured cells from WT and $B_1^{-/-}$ in the presence and absence of NEM, a CAT-1 blocker. WT cells showed a consistent ability to internalize L-Arg, reaching levels of 115 µg/mg of protein during the 15 h of incubation. Under the same conditions, $B_1^{-/-}$ cells exhibited significant lower levels of L-Arg uptake (84 µg/mg of protein). When pre-incubation was performed in the presence of NEM, L-Arg uptake was markedly reduced (50%) in WT cells. Otherwise, NEM had no significant effect on L-Arg uptake by $B_1^{-/-}$ cells. Results are shown in Figure **3**.

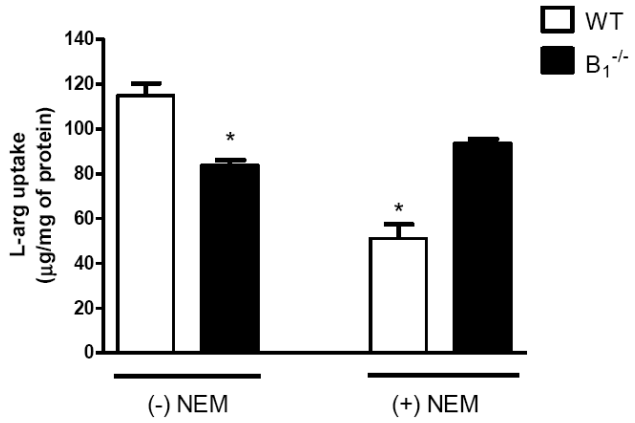

Figure 3. L-Arg uptake by WT and $B_1^{-/-}$ endothelial cells, measured in the culture medium after 15 h of incubation. Experiments were performed in the presence or absence of the CAT-1 blocker, NEM (1 mmol/L). Data are mean ± sem; n = 4; **P* <.05 *vs* WT.

3.3. CAT-1: mRNA LEVELS and Protein Expression in Endothelial Cells

To determine whether the observed changes in NO production and L-Arg uptake in $B_1^{-/-}$ cells could be associated with a possible alteration in the endothelial CAT-1 expression, we investigated the mRNA levels and protein contents through qPCR and western blot, respectively. In both investigations, CAT-1 expression was markedly decreased in $B_1^{-/-}$ cells. An approximate two-fold decrease in the mRNA levels for CAT-1 was detected in $B_1^{-/-}$ when compared to WT cells. In agreement, blots revealed a severe reduction in the protein CAT-1 expression in $B_1^{-/-}$ cells (Fig. **4**).

4. DISCUSSION

Several studies have demonstrated the influence of the kallikrein-kinin system in the vascular control. Kinin B_1 and B_2 receptors share low homology (36% identity at the amino acid sequence level), differ in their pharmacological profiles as well as in their expression patterns, and activate different intracellular pathways [1,2,19]. In addition, the B_2 receptor is rapidly desensitized and internalized, whereas the B_1 subtype is not and produces prolonged signaling [2]. While the B_2 receptor has been extensively investigated, the B_1-subtype is still poorly understood. The present study demonstrates that genetic deletion of the kinin B_1 receptor downregulates the expression of CAT-1, leading to impairment in L-Arg uptake and deficient NO release by endothelial cells.

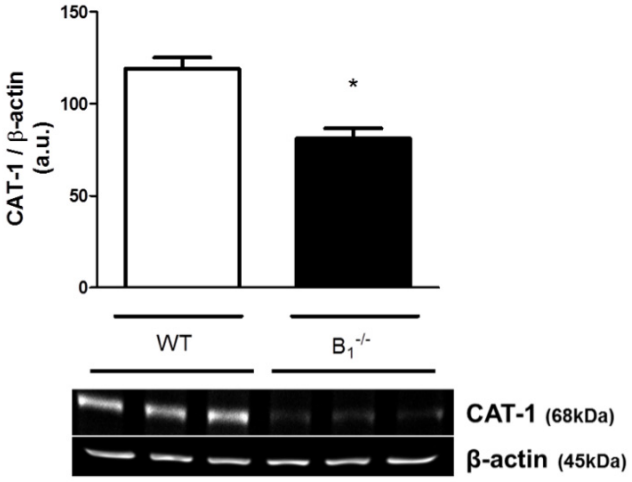

Figure 4. *Above:* Densitometric analysis of protein contents. *Bellow:* Representative western blot analysis showing CAT-1 protein levels in endothelial cells from WT and $B_1^{-/-}$. Data represent the mean ± sem; n = 3-4; **P* <.05 *vs* WT.

NO is a potent cell signaling and vasodilator molecule that plays important roles in several biological processes including the control of vascular tone. All isoforms of NOS utilize L-Arg as the substrate to synthetize NO. Concerning the vascular NO production, the most common concept is that the B_2 receptor signaling correlates to the Ca^{++}-dependent endothelial NOS (eNOS) activation, while B_1 is correlated to the Ca^{++}-independent inducible NOS (iNOS), after injury and/or inflammatory stimuli [19]. Nevertheless, B_1 receptor stimulation has also been shown to activate eNOS transfected into adventitial fibroblasts of cerebral arteries [20], and to stimulate NO release from eNOS in bovine pulmonary artery endothelial cells [21].

In the present study we investigated the ability of cultured endothelial cells obtained from $B_1^{-/-}$ mice in producing NO after stimulation with ACh, a strategic pharmacological tool widely employed to study vascular function through NO production via eNOS. Our results demonstrated that these cells were consistently responsive to ACh stimulation; however, NO release was significantly decreased in $B_1^{-/-}$ when compared to WT. This lower NO production exhibited by $B_1^{-/-}$ cells is in agreement with our previous observation in resistant arterioles of $B_1^{-/-}$ mice, where isolated vessels exhibit a severe loss in the responsiveness to ACh-induced relaxation [4]. Thus, the present results confirm that deletion of the B_1 receptor induces important alterations at the endothelial level, leading to impaired NO production and endothelial dysfunction.

Importantly, our present experiments showed that stimulation with the B_1 preferential agonist des-Arg^9-BK promoted a consistent NO release, in a similar manner to that obtained with ACh in the same cells. Therefore, in our cultured endothelial cells, B_1 expression occurs even in the absence of inflammatory stimuli. Indeed, several studies have demonstrated the constitutive expression of the B_1-subtype in different cells and tissues, including bovine aortic and pul-

monary artery endothelial cells [22-24] and abdominal aorta of mice [25]. The observation that the kinin B_1-subtype is not restricted to the inflammatory context might clarify novel aspects of this receptor in the vascular physiology.

It is widely accepted that a decreased bioavailability of vascular NO may be a consequence of a deficiency in NO production and/or an enhancement in NO degradation. Reduction in NO release may result from different factors, including decrease in the NOS activity [26] and deficient intracellular levels of its substrate L-arg [27]. Regarding the $B_1^{-/-}$ model, we have previously demonstrated that vascular NOS activity is not reduced in comparison to WT samples; instead, those experiments indicated a significant increase in the total NOS activity in the presence of substrate and cofactors supplementation [4]. Therefore, in the present study we employed cultured endothelial cells to analyze the consequences of the B_1 receptor deletion on the internalization rate of the NO precursor, L-Arg.

L-Arg is supplied by intracellular synthesis and by uptake from extracellular space. Interestingly, several studies indicate that NO production by endothelial cells can be increased by extracellular arginine, despite a saturating intracellular arginine concentration. This observation has been termed the "arginine paradox" and cannot be completely explained based on available data [28]. One paradigm that could explain this phenomenon is that in endothelial cells the intracellular arginine is sequestered in one or more pools that are poorly, if at all, accessible to eNOS, whereas extracellular arginine transported into the cell is preferentially delivered to eNOS [9]. Under this paradigm, the arginine internalization may represent a crucial event for the cellular physiology.

Several studies have demonstrated that supplementation of L-Arg improves vascular function, probably by increasing NO release [29,30]. Thus, in the present study we tested the effects of L-Arg supplementation in cultured endothelial cells obtained from $B_1^{-/-}$; however, no positive effect was detected at all. This result allowed us to hypothesize that the endothelial dysfunction and reduced NO levels observed in the $B_1^{-/-}$ mice [4] could be a consequence of a deficient uptake of L-Arg at the endothelial level. In order to test this hypothesis we firstly determined the ability of these cells to internalize L-Arg by measuring its contents in the culture medium through HPLC. L-Arg was quantified in medium samples collected before and after 15 h of cell incubation, since this time interval was found appropriate to determine consistent and reproducible L-Arg uptake levels in triplicate measurements, according to preliminary studies. Under this condition, L-Arg uptake was significantly decreased in $B_1^{-/-}$ in comparison to WT cells. Additionally, NEM reduced L-Arg uptake by 55% in WT, with no significant alterations in the values found for $B_1^{-/-}$ cells. Finally, we investigated the expression patterns of CAT-1 in endothelial cells through qPCR and western blot analysis. In both situations, CAT-1 expression was markedly reduced in $B_1^{-/-}$cells. Taken together, these results strongly support the concept that the deletion of B_1 receptor induces important alterations in NO metabolism and vascular physiology by modifying CAT-1 expression and reducing L-Arg internalization.

Our present results cannot explain whether there is an alternative mechanism used by $B_1^{-/-}$ cells to internalize L-Arg or not. However, one possibility is a differential regulation of the other carrier from system y^+ induced by B_1 receptor deletion. Changes in arginine uptake produced by different stimuli have been clearly associated with CAT-1/CAT-2 altered expression [13,31]. Similarly, the overexpression of CAT-2 and CAT-3 isoforms was demonstrated as a compensatory mechanism in embryonic fibroblast cells derived from CAT-1 knockout mice [32]. Thus, the occurrence of a similar phenomenon in $B_1^{-/-}$ endothelial cells cannot be discarded.

CAT-1 is expressed almost ubiquitously, but its protein expression patterns and regulation are not completely elucidated. Some regulators of CAT-1 mRNA levels have been identified, including cytokines, growth factors and hormones, working in a tissue-specific manner [7]. CAT-1 mediated L-Arg transport in endothelial cells has been shown to be subject to regulation by the activity of kinases such as p42/44 MAP kinase and the protein kinase C (PKC) family [12,33].

The subcellular location of CAT-1 in cells that use arginine for NO synthesis has been an interesting target for investigation, but the current data are not conclusive. At the endothelial level, CAT-1 was found to be soluble in triton and localized in clusters (not resembling caveoli), that were dispersed by disrupting microtubules [34]. In contrast, a later study reported CAT-1 to co-localize with eNOS at the caveolae [9], facilitating NO synthesis. According to these authors, CAT-1 and eNOS may be physically associated, forming a protein complex able to provide a mechanism for directed delivery of L-Arg to eNOS and NO synthesis [9]. Therefore, it has not yet been understood if this transport system consists of only the carrier protein itself, or also of additional regulatory proteins associated. Our present results open new approaches concerning these protein-protein interactions, and further studies are needed to elucidate the possible participation of the kinin B_1 receptor on CAT-1 / eNOS complex.

CONCLUSION

These findings suggest an important role of the kinin B_1 receptor in the vascular control by interfering with CAT-1 expression, L-Arg uptake and NO release in endothelial cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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