Cross talk between Toll-Like Receptor 4 (TLR4) and Proteinase Activated Receptor 2 (PAR-2) is involved in vascular function

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Running Title TLR-4 and PAR-2 cross-talk

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Summary

Background and purpose. PARs and TLRs receptors are involved in innate immune response. Aim of this study was to evaluate the possible cross-talk between PAR-2 and TLR4 in vascular district in physiological condition and how it varies following stimulation of TLR4 by using *in vivo* and *ex-vivo* models.

Experimental approach Thoracic aortas were harvested from both naïve and endotoxemic rats for *in vitro* studies. Arterial blood pressure was monitored in anesthetized rats *in vivo*. In aortic homogenates immunoprecipitation of TLR4 receptor followed by PAR-2 immunoblot and western blot analysis for TLR4 and PAR-2 were performed. LPS was used as TLR4 agonist while PAR2-AP was used as PAR2 agonist.

Key results PAR-2, but not TLR4, expression was enhanced in aorta of endotoxemic rats. PAR2-AP-induced vasorelaxation was increased in LPS-treated rats aortic rings. TLR4 inhibitors, curcumine and resveratrol, reduced PAR-2-AP-induced vasorelaxation and PAR-2-AP-induced hypotension in both naïve and endotoxemic rats. Immunoblotting with anti-PAR-2 of anti-TLR4 immunoprecipitates revealed PAR-2/TLR4 association in both naïve and endotoxemic rats. Western blot performed on surnatants of anti-TLR4 immunoprecipitates showed the presence of PAR-2 exclusively in aorta of endotoxemic rats. Finally, in $TLR4^{-/-}$ mouse aortic rings, PAR-2 message as well as PAR2-AP-induced vasodilatation, was impaired compared to wild type.

Conclusions and Implications PAR-2 and TLR4 are constitutively associated in vascular district and contribute to vascular homeostasis.

Introduction

Proteinase-activated receptors 2 (PAR-2) belongs to a seven transmembrane G-protein coupled receptors activated by proteolytic cleavage (Macfarlane et al., 2001; Hollenberg and Compton, 2002). All PARs share a unique mechanism of activation where the serine proteases cleave at specific sites within the extracellular N-terminus to unmask a tethered ligand domain that interacts with receptor in the extracellular loop II initiating signalling. This process is irreversible since once cleaved PARs, they cannot longer be used by the cell so they are degraded terminating signalling (Cottrell et al., 2003). Thus far, four PAR receptors have been described: PAR-1, PAR-2, PAR-3 and PAR-4 (Hollenberg, 1999). While the ligand of PAR-1, PAR-3 and PAR-4 has been identified in thrombin, PAR-2 has been described as activated by trypsin (Nystedt et al., 1994), tryptase (Molino et al., 1996), or factor Xa (Fox et al., 1997), but not thrombin (Cicala, 2002). PAR-2 can be activated by specific synthetic peptide (PAR2-AP) which, mimicking the specific tethered ligand sequence SLIGRL, can activate the receptor without causing proteolysis (Dery et al., 1998). In the last decade, we and others have demonstrated that PAR-2 is expressed in vascular district and, after exposure to lipopolysaccaride, it results up-regulated both *in vitro* and *in vivo* suggesting a possible role for PAR-2 in endotoxemia (Nystedt et al., 1997; Cicala et al., 1999; Morello et al., 2005). The notion that PAR-2 is over-expressed in inflammatory-based diseases is supported by literature, however it is not well established if this receptor has a protective or detrimental role (Bucci et al., 2005).

Recently a new emerging family of receptors has been identified as a front-line subsystem against invasive microorganisms for both innate and adaptive immunity namely Toll Like Receptors (TLR) (Iwasaki and Medzhitov, 2004). To date, 12 TLRs have been identified in both vertebrates and invertebrates (Hoffmann and Reichhart, 2002; Roach et al., 2002) and all of them are involved in triggering defensive antimicrobial immune responses (Akira and Hemmi, 2003; Akira et al., 2006; Zhang and Schluesener, 2006). TLRs receptors recognise specific motifs found in microorganisms,

but not in eukaryotes, designed as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002; Aderem and Ulevitch, 2000). Once activated by PAMPs, TLRs initiate a signal transduction cascade that leads to NF-kB activation with production of proinflammatory cytokines, such as tumor necrosis factor α, IL-1 and IL-6 (Barton and Medzhitov, 2004; Mitchell et al., 2007). In particular, TLR4 signal transduction has been recognized as a key pathway for lipid A moiety of LPS from Gram-negative bacteria inducing activation of various cells stimulating widespread inflammation and activating coagulation cascades (Lolis and Bucala, 2003; Nakamura et al., 2008; O'Neill and Bowie, 2007).

Recent studies place PARs receptors, together with TLRs and nucleotide-binding oligomerization domain (NOD) receptors, as a part of innate immune response. Indeed, a complex interplay of different receptors of these three families is required for appropriate innate immune responses to different types of bacteria; this bacterial-host communication leads several signal transduction pathways activation with consequent production of cytokines, antimicrobial peptides and apoptosis (Chung et al., 2010). In this context, it has been demonstrated that PAR-2 activation participates in the pathogenesis of periodontitis caused by *porphiromonas gingivalis* (Holzhausen et al., 2006), colitis caused by *citrobacter rodentium* (Hansen et al., 2005), and in infections sustained by *serratia marcescens* (Kida et al., 2007) and *pseudomonas* aeruginosa (Kida et al., 2008). Moreover, it has been shown that in transiently PAR-2 transfected HEK-293T cells a synergistic action between PAR-2 and TLR4 takes place, suggesting a cooperation of these two receptors in NF-κB-mediated inflammatory response (Rallabhandi et al., 2008). This literature clearly suggests interplay between PAR-2 and TLR4 in infection-based inflammatory disease, where PAR-2 and TLR4 activation is enhanced and their cooperation is valuable. However, the possible PAR-2/TLR4 crosstalk in physiological environment is less investigated. Aim of this study was to evaluate the possible cross-talk between PAR-2 and TLR4 in vascular district in physiological condition and how it varies following stimulation of TLR4 by using *in vivo* and *ex-vivo* models.

Materials and methods

Animals

Male C57BL/10ScN mice and male Wistar rats were purchased from Harlan, Italy Male C57BL/10Cr ($TLR4^{-/-}$) mice were a generous gift of Prof. S. Cuzzocrea, University of Messina, Italy. All mice used for experiments were 6-8 week of age. TLR4 KO mice were homozygous for a 74-kb genomic deletion encompassing *Tlr4* that resulted in absence of both mRNA and protein and that make them refractory to the biological activity of LPS (Poltorak et al., 1998; Poltorak et al., 2000). All animal procedures were performed according to the Declaration of Helsinki of the European Community guidelines for the use of experimental animals and authorised by Centro Servizi Veterinari Università degli Studi di Napoli "Federico II".

Reagents

PAR-2–activating peptide (PAR2-AP; SLIGKV-NH_2) was synthesized by standard solid-phase 9-fluorenylmethoxycarbonyl (FMOC) chemistry with an automated peptide synthesizer (Applied Biosystems, model 432A). Peptide was purified by reverse-phase high-performance liquid chromatography, and its identity was confirmed by mass spectroscopy, as described previously [11]. Urethane, heparin, resveratrol (RSV), curcumine (CRC), acetylcholine (Ach), phenylephrine (PE), dimethylsulfoxide (DMSO), superoxide dismutase (SOD) and lipopolysaccharide (LPS) from Escherichia coli 0.127:B8 were purchased from Sigma Chemical Co (Milan, Italy). Chlorisondamine (Chl) was purchased from Tocris Cookson (Avon, England).

Blood Pressure Measurement

Male Wistar rats (Harlan, Italy) weighing 250-300g were anesthetized with urethane (solution 15% wt/vol; 1.5 g/kg ip.) Once anesthetised, rats were pre-treated with the irreversible ganglion-blocking agent, chlorisondamine (2.5 mg/kg i.p.) in order to eliminate any influence of autonomic nervous

system activation on changes in mean arterial blood pressure (MABP) induced by PAR-2AP (Cicala et al., 2001). The right jugular vein and the left carotid artery were cannulated for drug administration and blood pressure measurement, respectively. The left carotid artery was connected to a pressure transducer (Ugo Basile, Comerio, Italy) and changes in arterial blood pressure were recorded continuously with a computerized system (Biopac System TCI 102). CRC (100mg/kg) or RSV (30 mg/kg) were administered by gavages 1 hour before PAR-2AP intravenous injection. After surgery, arterial blood pressure was allowed to stabilize for about 30 minutes. Once the blood pressure was stable PAR2-AP (0.3 mg/kg) or vehicle (saline) was administered i.v. every 20 minutes for 3 times consecutively and blood pressure monitored for a total time of 60 minutes. The change in MABP, as previously described, (Cicala et al., 1999) was characterized by a rapid fall lasting 1 minute. There was no tolerance to the hypotensive effect of PAR-2AP (data not shown), Groups of 6 rats for each treatment were used. Blood pressure values were expressed as MABP calculated as area under the curve obtained following PAR2-AP administration. The MABP values were monitored every 10 seconds up to 1 minute.

Ex vivo studies

Male Wistar rats (Harlan, Italy) weighing 250-300g (n=4 for each group of treatment) were anaesthetized with enflurane, then LPS ($13.5x10^6$ U/kg), or an equal volume of saline, was intravenously injected through the caudal vein. The dose of LPS was chosen from a previous study (Cicala et al., 1999). After 4 and 8h from LPS or saline administration, animals were sacrificed and thoracic aorta was rapidly harvested, dissected, and cleaned of adherent connective and fat tissue. Rings of 2-3 mm length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O_2 -5% CO_2) Krebs solution maintained at 37°C. The rings were connected to an isometric transducer (type 7006, Ugo Basile, Comerio, Italy) and changes in tension were recorded continuously with a computerized system (Data Capsule 17400, Ugo Basile, Comerio, Italy). The composition of the Krebs solution was as follow (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5,

$NaHCO_3$ 25, and glucose 10.1. The rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min, during which tension was adjusted, when necessary, to 0.5g and bathing solution was periodically changed. In each experiment, rings were first challenged with PE (1 µM) until the responses were reproducible. To verify endothelium integrity, Ach cumulative concentration-response curve (10 nM – 30 µM) was performed on PE-precontracted rings. The rings were then washed and contracted with PE (1 µM) and, once a plateau was reached, a cumulative concentration-response curve of PAR2-AP (10 nM – 30 µM) was performed. A preliminary study on the optimal incubation time and concentration of the drug treatments was carried out (data not shown). CRC (10 µM; 15 minutes), RSV (10 µM; 15 minutes), SOD (300U/ml; 15 minutes) or vehicle (DMSO) were added in the organ baths; then the rings were contracted with PE (1 µM) and a cumulative concentration–response curve to PAR2-AP was performed.

In another set of experiments, a cumulative concentration-response of PAR-2AP was carried out on aortic rings harvested from both $TLR4^{-/-}$ and C57BL/10ScN.

Quantification by Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The presence of PAR-2 mRNA was determined by quantitative PCR. Briefly, total RNA from omogenated aorta tissue was extracted by using TRIzol reagent (Invitrogen, Milan, Italy), subsequently to eliminate genomic DNA contamination 1 µg of above RNA was treated with RQ1 RNase-free DNase I (Promega Corporation, Madison, USA) and reverse transcription was performed using M-MLV Reverse Transcriptase (Invitrogen, Milan, Italy) according to the manufacturer's recommendations. Real-time RT-PCR was carried out with cDNAs by using Sybr Green PCR Master Mix (Applied Biosystems, Monza, Italy) and 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). All cDNA samples were run in triplicate in 25-µL

reactions. Primers used were specifically designed using Primer Express Software 2.0 (Applied Biosystems) and validated for their specificity: FW:5'-CCGGGACGCAACAACAGTA-3' RV:5'-TTCCCAGTGATTGGAGGCTG -3product length = 71. GAPDH was used as internal control.

Samples were incubated at 50°C for 2 min and at 95°C for 10 min followed by 40 cycles at 95 °C for 15 s and 60°C for 1 min. Differences in cDNA input were corrected by normalizing signals obtained with primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To exclude nonspecific amplification and/or the formation of primer dimers, control reactions were performed in the absence of target cDNA. Gene expression levels were calculated using the $2^{-\Delta CT}$ method and are presented as ratio between mean fold change of target gene and GAPDH ± standard error.

Western Blotting

Aortic tissue of naive and LPS treated rats, $TLR4^{-/-}$ and respective wild type strain C57BL/10ScN were homogenized in modified RIPA buffer (Tris HCl 50mM, pH 7.4, tritonX-100 1%, Sodium-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, phenylmethanesulphonylfluoride 1 mM, aprotinin 10µg/ml, leupeptin 20mM, NaF 50 mM) using a polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation of homogenates at 12000 r.p.m. for 15 min, protein concentration was determined by Bradford assay using BSA as standard (Bio-Rad Laboratories, Milan, Italy). 30µg of the denatured proteins or 40µg of surnatants obtained from immunoprecipitates were separated on 10% or 8% SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in PBS-tween 20 (0.1 %, v/v) containing 5% non fat dry milk for 1 hour at room temperature, and then incubated with anti-PAR2 (1:500) or anti-TLR4 (1:1000) overnight at 4°C. The filters were washed with PBS-tween 20 (0.1%, v/v) extensively for 30 min, before incubation, for 2 hours at 4°C, with the secondary antibody (1:5000) conjugated with horseradish peroxidase antimouse IgG or antirabbit IgG, respectively. The membranes were then

washed and immunoreactive bands were visualized using an Enhanced Chemiluminescence Substrate (ECL; Amersham Pharmacia Biotech, San Diego, CA, USA).

TLR 4 immunoprecipitation and PAR-2 immunoblotting

All steps were performed at 4°C. Aortas, prepared from LPS-treated or saline-treated rats were washed with Krebs solution and homogenized in modified RIPA buffer (50 mM Tris -HCI pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM PMSF; 10 μg/ml aprotinin, 20 mM leupeptin, 50 mM NaF). After 30 min, homogenates were centrifuged for 10 min at 13000 r.p.m. in an eppendorf microfuge in order to remove nuclei and cell debris, and 300 μl of the resulting supernatant (containing 400 μg of cleared lysate) were incubated overnight with the rabbit polyclonal anti-TLR 4 antibody [TLR4 (M300) sc-30002, Santa Cruz Biotechnology, Inc., Santa Cruz, CA] or normal rabbit serum (to evaluate non-specific binding) on a rotating wheel. The antigen-antibody complexes were incubated for 2h on a rotating wheel with protein A/G – plus Agarose (sc-2003, Santa Cruz Biotechnology, Inc.). After centrifugation at 600rpm for 15 seconds, the surnatants were collected, transferred into eppendorfs and conserved at -80°C for western blot analysis. The bound complexes were washed once with lysis buffer, twice with buffer A (10 mM Tris-HCI pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P40), twice with buffer B (10 mM Tris-HCI pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P40) and once with 10 mM Tris-HCI pH 7.4. The surnatants of the beads were collected and immunoprecipitated material was eluted from the beads by boiling in Laemmli sample buffer and subjected to SDS-PAGE (8%). The blot was performed by transferring proteins from a gel to PVDF membrane at 250 mA for 40 min at room temperature. The filter was then blocked with 1x PBS, 5% non fat dried milk for 1 h at room temperature and probed with monoclonal antibody anti-PAR-2 [1:500, PAR-2 (SAM11) sc-13504, Santa Cruz, Inc.] dissolved in 1x PBS, 5% non fat dried milk at 4°C, overnight. The secondary antibody (anti mouse IgG-horseradish peroxidase conjugate 1:5000 dilution) was incubated for 2 h

at 4°C. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, Piscataway, NJ) according to the manufacturer's instructions, and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY, United States). A protein band of about 40 kDa on x-ray film was evidenced.

Statistical analysis

Data were expressed as mean ± s.e.m. Statistical analysis was determined by using one or two way ANOVA followed by Dunnett's or Bonferroni's test for multiple comparisons, respectively, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when p was less than 0.05.

Results

PAR2-AP-induced vasorelaxation is increased in aortic rings from LPS treated rat

As already previously shown (Cicala et al.,1999), western blot analysis revealed an enhanced expression of PAR-2 receptor following LPS injection when compared with vehicle at all times tested (supplemental figure 1A). PAR2-AP induced vasorelaxation was increased in a time dependent manner in rat aortic rings harvested 4 and 8h following LPS administration (supplemental figure 1B). Conversely, TLR4 expression was not modified after LPS treatments, at all times tested (supplemental figure 1C)

TLR4 inhibitors, curcumin (CRC) and resveratrol (RSV), reduced PAR2-AP-induced relaxation in naive and LPS-treated rats

In order to investigate on crosstalk between PAR-2 and TLR4 signalling, aortic rings were treated with two different compounds recently identified as inhibitors of TLR4 pathway i.e. curcumin (CRC) and resveratrol (RSV). Indeed, it has been shown that CRC inhibits both ligand-induced (MyD88 dependent pathway) and ligand-independent (MyD88 independent, TRIF-dependent pathway) dimerization of TLR-4 (Youn et al., 2006; Son et al., 2008; Lubbad et al., 2009), while RSV inhibits specifically the MyD88 independent TRIF-dependent pathway (Son et al., 2008; Lubbad et al., 2009; Youn et al., 2005). In order to verify the specificity of CRC and RSV, the maximal concentrations of either CRC (30μM) or RSV (10μM) have been tested on Ach-induced vasodilatation. As shown in figure 3, both compounds did not affect Ach –induced vasodilatation.

Both CRC (3, 10, 30 μM) and RSV (1, 3, 10 μM) significantly and in a concentration–dependent manner reduced PAR2-AP-induced vasorelaxation in naïve rats (figures 1A, 2A). In LPS-treated rats CRC (figure 1 panels B and C) as well as RSV (figure 2 panels B and C) significantly inhibited PAR2-AP-induced vasorelaxation at the different time points tested as well. The inhibition observed following CRC and RSV pre-treatment on PAR2-AP-induced vasorelaxation was not significant different between naïve and endotoxemic rats. Moreover, to exclude the possibility that

the effect of CRC and RSV on PAR2-AP-induced vasorelaxation was imputable to their anti-oxidant properties, we pre-treated aortic rings with superoxide dismutase (SOD, 300U/ml) at a concentration known to act as superoxide anion scavenger. SOD did not modify the PAR2-AP-induced vasorelaxation (EC_{50} = $3.98x10^{-6}$ M and $3.80x10^{-6}$ M in presence of vehicle and SOD, respectively, data not shown).

CRC and RSV reduced PAR-2AP-induced hypotension

In order to prove that hypotension induced by selective activation of the PAR-2 receptor involves TLR4 signalling, CRC (100 mg/kg) and RSV (30 mg/kg) were administered prior to PAR2-AP intravenous administration. Both CRC and RSV inhibited PAR2-AP-induced hypotension *in vivo* in naïve rats (figure 4).

Physical interaction between PAR-2 and TLR4 in naive and LPS-treated rats

To further gain insights into the molecular mechanism of PAR-2/TLR4 crosstalk an immunoprecipitation study was carried up. In homogenates of aortas harvested from both naïve and LPS-treated rats, immunoprecipitation of TLR4 receptor followed by PAR-2 immunoblot was performed. Immunoblotting with anti-PAR-2 of anti-TLR4 immunoprecipitates revealed that PAR-2 was associated with TLR4 receptor, since a 40-kDa band consistent with PAR-2 core protein (Gruber et al., 2004) was shown (figure. 5B lane B). The complex between PAR-2 and TLR4 receptor was also evident after *in vitro* PAR2-AP stimulation of aortas (figure 5B lane D), suggesting that PAR-2 was associated with TLR4 prior to, as well as, after receptor activation. Similar findings have been also obtained in aortas harvested by endotoxemic animals (figure 5B lane E-H). To evaluate the specificity of the 40-kDa band, cell lysate of aortas stimulated or not with PAR-2 from control and endotoxemic rats was incubated with the corresponding normal rabbit serum instead of anti-TLR-4 antibody (figure 5B lanes A, C, E, and G). Analysis of western blot

performed on surnatants obtained from anti-TLR4 immunoprecitates revealed the presence of PAR2 receptor exclusively in aorta harvested from LPS-treated rats (figure 5C).

PAR-2 expression was increased in TLR4$^{-/-}$ mice

In order to confirm the cooperation between PAR-2 and TLR4 receptors, we performed functional study on TLR4$^{-/-}$ mice. In isolated aortic rings of these mice both Ach-induced vasodilatation and the endothelium-independent dilator agent SNP, caused a similar degree of response in TLR4$^{-/-}$ and wt mice (figure 6A-B). RT-PCR analysis revealed a reduction of PAR-2 expression in aorta of TLR4$^{-/-}$ mice compared to wt (fig. 7A). This finding is confirmed in the functional study where , PAR2-AP-induced vasorelaxation was significantly reduced in aortic rings harvested from TLR4$^{-/-}$ mice compared to WT (fig. 7C). Western blot analysis for PAR-2 did not reveal any significant difference between TLR4$^{-/-}$ and wt mice (7B).

Discussion

We have previously shown that PAR-2 is involved in LPS induced hypotension, however we could not define the transduction mechanism underlying PAR-2 effect in septic shock (Cicala et al., 1999). At the time it was not known that LPS explicates its action mainly through TLR4 receptor. Following recent evidence suggesting a possible cooperation among PAR receptors in TLRs signalling [Rallabhandi et al., 2008; Moretti et al., 2008; Uehara et al., 2008), here we have sought to investigate a possible cross-talk between PAR-2 and TLR4 in the vascular district. To pursue this goal we have used two different approaches: the first *ex vivo* by operating a pharmacological modulation on isolated aortic rings, harvested from naïve and endotoxemic rats. The second *in vivo*, using PAR2-AP-induced hypotension in anesthetised rats in order to validate the data obtained *ex vivo*. The rationale in using aorta harvested from LPS-treated rats relies on the finding that LPS injection induced an increase of PAR2 expression in vascular tissue.

First we have evaluated if RSV or CRC, two recently recognised TLR4 antagonists (Youn et al., 2006; Son et al., 2008; Lubbad et al., 2009; Youn et al., 2006), could reduce PAR-2AP-induced vasorelaxation. Both compounds significantly inhibited PAR2-AP-induced vasodilatation in aortas harvested from either LPS-treated or control rats without affecting NO release. This lack of difference in activity of TLR4 inhibitors on both control and LPS rats suggested that a constitutive interaction may exists between PAR-2 and TLR4. This latter result taken together with the finding that PAR-2, but not TLR4, expression is increased in aortic tissue harvested from rats injected with LPS at 4h and 8h, indicate that the PAR-2 over-expressed following LPS treatment is not physically associated to TLR4. Immunoprecipitation study reveals that PAR-2 physically interacts with TLR4 in normal conditions and that LPS treatment does not increase this interaction. These data confirm that PAR-2/TLR4 association is constitutive in the vascular tissue and LPS-induced PAR-2 over-expression does not involve a further increase of interaction with TLR4. Western blot analysis for PAR-2 receptor performed on supernatants obtained from anti-TLR4 immunoprecipitates shows

that a significant amount of PAR-2 exceeded exclusively following LPS treatment. Therefore the over expressed PAR-2 is not bound to TLR4.

In order to test if this is also the case *in vivo,* we treated naïve rats with RSV or CRC and then we induced hypotension by a bolus injection of PAR-2AP. CRC and RSV significantly reduced PAR2-AP-induced hypotension by using a dose of these inhibitors known to act as TLR-4 inhibitor as opposed to higher doses (Bengmark, 2006; Silan, 2008). This data suggests the involvement of TLR4 signalling in PAR-2 activation. The fact that RSV, the MyD88 independent TRIF-dependent pathway inhibitor, resulted more potent than CRC both in *ex vivo* and *in vivo* experiments suggests that PAR-2 activation might involve this specific TLR4 signalling.pathway This mechanism has been already proposed by Rallabhandi and colleagues by using transiently PAR-2 transfected HEK-293T cells (Rallabhandi et al., 2008). Thus, data obtained *in vivo* are confirmatory of the *ex vivo* experiments e.g. that PAR-2/TLR4 cross-talk is involved in the modulation of the vascular tone in physiological conditions. When a pathological stimulus occurs i.e. LPS injection, there is an over-expression of PAR-2 that is no more related to TLR4.

The experiments carried out on $TLR4^{-/-}$ mice provide a proof of concept of this crosstalk between PAR2 and TLR4 receptors. RT-PCR analysis performed on aorta harvested from $TLR4^{-/-}$ mice showed a reduction of PAR-2 expression. Similarly, functional study showed a significant reduction of PAR2-AP-induced vasodilatation. In order to assess the vascular reactivity of $TLR4^{-/-}$ mice we have checked both endothelium-dependent (with Ach) and endothelium-independent (with SNP) vasodilatation that resulted not significantly different from wild type mice. These data indicate that, in absence of TLR4, PAR-2 signalling is impaired most likely because of the lack of PAR-2/TLR4 cooperation.

Conclusions

Our study demonstrates that in vascular district PAR-2 and TLR4 cooperate in terms of molecular signalling both in physiological or pathological condition. LPS priming causes an overexpression of

PAR-2 exceeding the TLR4 binding capability. This finding shades a new light on the dual role of PAR-2 in physiological versus pathological conditions. Indeed, it could be assumed that when the amount of PAR-2 expressed exceeds TLR4 capability, PAR-2 may explicate a detrimental effect. Conversely, when all PAR-2 expressed is bound to TLR4 it can explicate a physiological beneficial effect. Our finding could explain why in some cases activation of PAR-2 is anti-inflammatory (Morello et al., 2005; Fiorucci et al., 2001) while in others is pro-inflammatory (Ferrell et al., 2003; Fiorucci et al., 2001; Hyun et al., 2008) and contributes to unravel the complex role played by this protease-activate receptor in cardiovascular homeostasis.

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Conflict of interest: none declared

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Legends:

Figure 1 (A) PAR-2AP-induced vasorelaxation was significantly and concentration dependently inhibited by CRC in aortic rings harvested from control rats (*** = $p<0.001$ vs. vehicle; two-way ANOVA). CRC (10 μM) pre-treatment affected PAR-2AP vasorelaxation in aortic rings harvested from LPS at **(B)** 4h (*** = $p<0.001$ vs. vehicle) and (**C**) 8h (*** = $p<0.001$ vs. vehicle; for each set of experiments were used n= 5 rats).

Figure 2 (A) PAR-2AP-induced vasorelaxation was significantly and concentration dependently inhibited by RSV in aortic rings harvested from control rats (*** = $p<0.001$ vs. vehicle; two-way ANOVA). RSV (10 μM) pre-treatment affected PAR-2AP vasorelaxation in aortic rings harvested from LPS at **(B)** 4h (*** = $p<0.001$ vs. vehicle) and (**C**) 8h (*** = $p<0.001$ vs. vehicle; for each set of experiments n=5 rats).

Figure 3 Pre-incubation of aortic rings with CRC and RSV did not affect Ach-induced vasodilatation. For each set of experiments were used n=4 rats).

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Figure 4 PAR-2AP-induced hypotension (0.3 mg/kg i.v.) was significantly reduced in control rats treated either with **(A)** CRC or with **(B)** RSV (*$p<0.05$; one way ANOVA, n= 4 for each treatment). Values were expressed as area under the curve (mm^2).

Figure 5 (A) Physical association of PAR-2 with TLR4. Anti PAR-2 immunoblot of antiTLR4 (lanes B, D, F and H) and normal rabbit serum control (lanes A, C, E and G) immunoprecipitation from lysates of aortas incubated in absence (lanes A, B, E and F) and presence of PAR-2AP (lanes C, D, G and H) in naïve (lanes A, B, C, D) and endotoxemic (lanes E, F, G, H) rats (n= 3 experiments). **(B)** Western blot analysis for PAR2 receptor performed on supernatants of TLR4

immunoprecipitates showed the presence of PAR2 receptor exclusively in aorta harvested from LPS-treated rats (n=3 experiments).

Figure 6: Ach-induced vasodilatation (A), as well as SNP-induced vasodilatation **(B),** were not significantly different in isolated aortic rings harvested from $TLR4^{-/-}$ mice when compared to wild type littermates, n= 10 for each group of animals.

Figure 7 (A) RT-PCR analysis performed on aortas harvested from $TLR4^{-/-}$ and wild type mice showed a reduction of PAR2 message in $TLR4^{-/-}$ mice (n=3 experiments). **(B)** Western blot analysis for PAR-2 receptor performed on lysates of aortas harvested from $TLR4^{-/-}$ and wild type mice did not reveal significant difference between the strain (n=3 experiments). **(C)** PAR-2AP-induced vasorelaxation was significantly impaired in aortic rings harvested from $TLR4^{-/-}$ (***= $p<0.001$, n=7 for each strain).

Supplemental Figure 1 (**A**) Western blot analysis shows a significant increase in PAR-2 expression in LPS (13.6U/kg) treated rats. (**B**) PAR-2AP-induced vasodilatation was significantly increased in aortic rings harvested from LPS-treated rats (after 4 and 8h from LPS) * = $p<0.05$ vs. saline ***= $p<0.001$ vs. saline; n = 10 for each group. **(C)** Western blot analysis revealed no difference of TLR-4 expression in LPS-treatment compared to naive rats.