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RESEARCH PAPER

Cross-talk between toll-like receptor 4 (TLR4) and proteinase-activated receptor 2 (PAR_2) is involved in vascular function

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BACKGROUND AND PURPOSE

Proteinase-activated receptors (PARs) and toll-like receptors (TLRs) are involved in innate immune responses. The aim of this study was to evaluate the possible cross-talk between PAR_2 and TLR4 in vessels 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 endotoxaemic rats for *in vitro* studies. Arterial blood pressure was monitored in anaesthetized rats *in vivo*. LPS was used as a TLR4 agonist while PAR_2 activating peptide (AP) was used as a PAR_2 agonist. Aortas harvested from $TLR4^{-/-}$ mice were also used to characterize the PAR_2 response.

KEY RESULTS

PAR_2, but not TLR4, expression was enhanced in aortas of endotoxaemic rats. PAR_2AP-induced vasorelaxation was increased in aortic rings of LPS-treated rats. TLR4 inhibitors, curcumine and resveratrol, reduced PAR_2AP-induced vasorelaxation and PAR_2AP-induced hypotension in both naïve and endotoxaemic rats. Finally, in aortic rings from $TLR4^{-/-}$ mice, the expression of PAR_2 was reduced and the PAR_2AP-induced vasodilatation impaired compared with those from wild-type mice and both resveratrol and curcumine were ineffective.

CONCLUSIONS AND IMPLICATIONS

Cross-talk between PAR_2 and TLR4 contributes to vascular homeostasis.

Abbreviations

Chl, chlorisondamine; CRC, curcumine; MABP, mean arterial blood pressure; PAR_2AP, protease activated receptor-2 activating peptide; PE, phenylephrine; RSV, resveratrol; TLR4, toll like receptor-4

Introduction

Proteinase-activated receptor 2 (PAR_2) is a member of the seven-transmembrane GPCR superfamily 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 the receptor in the extracellular loop II initiating signalling. This process is irreversible because once cleaved, PARs can no longer be used by the cell, so they are degraded and signalling is terminated (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 for PAR_1, PAR_3 and PAR_4 has been identified as thrombin, PAR_2 can be activated by trypsin (Nystedt *et al.*, 1994), tryptase (Molino *et al.*, 1997) and factor Xa (Fox *et al.*, 1997), but not thrombin (Cicala, 2002). PAR_2 can be activated by a specific synthetic peptide [PAR_2 activating peptide (AP)], which, by 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 tissues and exposure to LPS results in it being up-regulated both *in vitro* and *in vivo*, suggesting a possible role for PAR_2 in endotoxaemia (Nystedt *et al.*, 1996; Cicala *et al.*, 1999; Morello *et al.*, 2005). The notion that PAR_2 is overexpressed in inflammatory-based diseases is supported by the literature; however, it has not been well established whether 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.*, 2005), 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 recognize specific motifs found in microorganisms, but not in eukaryotes, designed as pathogen-associated molecular patterns (PAMPs) (Aderem and Ulevitch, 2000; Janeway and Medzhitov, 2002). Once activated by PAMPs, TLRs initiate a signal transduction cascade that leads to NF-κB activation with the production of pro-inflammatory cytokines, such as TNF-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 the lipid A moiety of LPS, from gram-negative bacteria, to induce activation of various cells stimulating widespread inflammation and activating coagulation cascades (Lolis and Bucala, 2003; O'Neill and Bowie, 2007; Nakamura *et al.*, 2008).

Recent studies have demonstrated that PARs, together with TLRs and nucleotide-binding oligomerization domain (NOD) receptors, are a part of the innate immune response. Indeed, a complex interplay between the different receptors of these three families is required for appropriate innate immune responses to different types of bacteria; this bacterial–host communication leads to the activation of several signal transduction pathways 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 HEK293T cells, a synergistic action between PAR_2 and TLR4 takes place, suggesting cooperation between these two receptors in NF-κB–mediated inflammatory responses (Rallabhandi *et al.*, 2008). These findings clearly suggest interplay between PAR_2 and TLR4 in infection-based inflammatory diseases, where PAR_2 and TLR4 activation is enhanced, and their cooperation is valuable. However, the cross-talk between $PAR_2$2 and TLR4 in normal physiological environments has been less well investigated. Hence, the aim of this study was to use *in vivo* and *ex vivo* models to evaluate any possible cross-talk between PAR_2 and TLR4 in vascular tissues in physiological conditions and how it varies following stimulation of TLR4.

Methods

Animals

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Male C57BL/10ScN mice and male Wistar rats (total number of rats used = 50) were purchased from Harlan, Udine, Italy. Male C57BL/10Cr ($TLR4^{-/-}$) mice were a generous gift from Prof. S. Cuzzocrea, University of Messina, Italy. All mice used for the experiments (total number used = 60) were 6–8 weeks of age. Animals were kept at a temperature of 23 ± 2°C, humidity range of 40 to 70% and 12h light/dark cycles. Food and water were available *ad libitum*.

$TLR4^{-/-}$ mice were homozygous for a 74-kb genomic deletion encompassing *Tlr4* that resulted in the absence of both its mRNA and protein and made them refractory to the biological activity of LPS (Poltorak *et al.*, 1998; 2000). All animal procedures were performed according to the Declaration of Helsinki of the European Community guidelines for the use of experimental animals and authorized by Centro Servizi Veterinari Università degli Studi di Napoli 'Federico II'.

Reagents

PAR_2AP (SLIGKV-NH_2) was synthesized by standard solid-phase 9-fluorenylmethoxycarbonyl chemistry with an automated peptide synthesizer (model 432A, Applied Biosystems, Carlsbad, CA, USA). Peptide was purified by reverse-phase HPLC, and its identity was confirmed by MS, as described previously. Urethane, heparin, resveratrol (RSV), curcumine (CRC), ACh, phenylephrine (PE), DMSO, SOD and 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) weighing 250–300 g were anaesthetized with urethane (solution 15% $wtvol^{-1}$; 1.5 $g{\cdot}kg^{-1}$ i.p.). depth anaesthesia was assessed by checking both abdominal and pedal withdrawal reflex throughout the duration of the experiment. Once anaesthetized, rats were pretreated with the irreversible ganglion-blocking agent, Chl (2.5 $mg{\cdot}kg^{-1}$ 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; Ugo Basile). CRC (100 mg·kg^{-1}) or RSV (30 mg·kg^{-1}) were administered by oral gavage 1 h before an i.v. injection of PAR_2AP. After surgery, arterial blood pressure was allowed to stabilize for about 30 min. Once the blood pressure was stable, PAR_2AP (0.3 mg·kg^{-1}) or vehicle (saline) was administered i.v. every 20 min for three consecutive times, and blood pressure was monitored for a total time of 60 min. The change in MABP, as previously described, (Cicala *et al.*, 1999) was characterized by a rapid fall lasting 1 min. There was no tolerance to the hypotensive effect of PAR_2AP (data not shown). Groups of six rats for each treatment were used. Blood pressure values were expressed as MABP calculated as area under the curve obtained following PAR_2AP administration. The MABP values were monitored every 10 s up to 1 min.

Ex vivo *studies*

Male Wistar rats (Harlan) weighing 250–300 g (n = 4 for each group of treatment) were anaesthetized with enflurane, then LPS (13.5×10^6 U·kg^{-1}) or an equal volume of saline, was injected i.v. through the caudal vein. The dose of LPS was chosen from a previous study (Cicala *et al.*, 1999). Four and 8 h after LPS or saline administration, the animals were killed and thoracic aortas 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) and changes in tension were recorded continuously with a computerized system (Data Capsule 17400, Ugo Basile). 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 0.5 g 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, a cumulative concentration–response curve to ACh (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 to PAR_2AP (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 min), RSV (10 μM; 15 min), SOD (300 U·mL^{-1}; 15 min) 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 PAR_2AP was performed.

In another set of experiments, a cumulative concentration–response curve to PAR_2AP, in the presence of CRC (10 μM; 15 min), RSV (10 μM 15 min) or vehicle (DMSO) was carried out on aortic rings harvested from both TLR4$^{-/-}$ and C57BL/10ScN mice. Data are expressed as % vasodilatation induced by the stimulus, when applied after a stable contraction had been obtained to 1 μM PE.

Quantification by real-time quantitative reverse transcriptase PCR (RT-PCR)

The presence of PAR_2 mRNA was determined by quantitative PCR. Briefly, total RNA from tissues of naive and LPS-treated rats, and naïve and LPS TLR4$^{-/-}$ and respective wild-type strain C57BL/10ScN was extracted by using TRIzol reagent (Invitrogen, Milan, Italy). Subsequently, to eliminate genomic DNA contamination, 1 μg of the above RNA was treated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI, USA) and reverse transcription was performed using M-MLV Reverse Transcriptase (Invitrogen) 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, USA). 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'-TTCCCAGTGA TTGGAGGCTG-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 GAPDH. To exclude non-specific 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 (Livak and Schmittgen, 2001) and are presented as ratio between mean fold change of target gene and GAPDH ± SEM.

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 50 mM, pH 7.4, tritonX-100 1%, sodium deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, PMSF 1 mM, aprotinin 10 μg·mL^{-1}, leupeptin 20 mM, NaF 50 mM) using a polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation of homogenates at 9193× g for 15 min, protein concentration was determined by the Bradford assay using BSA as standard (Bio-Rad Laboratories, Milan, Italy); 30 μg of the denatured proteins or 40 μg of supernatants 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^{-1}) containing 5% non-fat dry milk for 1 h at room temperature, and then incubated with anti–PAR_2 (1:500) or anti-TLR4 (1:1000) overnight at 4°C. The filters were washed with PBS-Tween 20 extensively for 30 min, before incubation, for 2 h at 4°C, with the secondary antibody (1:5000) conjugated with HRP antimouse IgG or antirabbit IgG, respectively. The membranes were then washed and immunoreactive bands were visualized using an Enhanced Chemiluminescence Substrate (Amersham Pharmacia Biotech, San Diego, CA, USA).

TLR4 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 $\mu g \cdot mL^{-1}$ aprotinin, 20 mM leupeptin, 50 mM NaF). After 30 min, homogenates were centrifuged for 10 min at 10789× *g* 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-TLR4 antibody [TLR4 (M300) sc-30002, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] or normal rabbit serum (to evaluate non-specific binding) on a rotating wheel. The antigen–antibody complexes were incubated for 2 h on a rotating wheel with protein A/G – plus Agarose (sc-2003, Santa Cruz Biotechnology, Inc.). After centrifugation at 23× *g* for 15 s, the supernatants 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 supernatants 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 1× 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 1× PBS, 5% non-fat dried milk at 4°C, overnight. The secondary antibody (anti-mouse IgG HRP 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, USA) according to the manufacturer's instructions, and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY, USA). A protein band of about 40 kDa on X-ray film was obtained.

Statistical analysis

Data are expressed as mean ± SEM. 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, USA). Differences were considered statistically significant when *P* was less than 0.05.

Results

PAR_2AP–induced vasorelaxation is increased in aortic rings from LPS-treated rats

As already previously shown (Cicala *et al.*, 1999), Western blot analysis revealed an enhanced expression of the PAR_2 receptor following LPS injection when compared with vehicle at all times tested (Supporting Information Figure S1A). PAR_2AP–induced vasorelaxation was increased in a time-dependent manner in rat aortic rings harvested 4 and 8 h following LPS administration (Supporting Information Figure S1B). Conversely, TLR4 expression was not modified after LPS treatments, at all times tested (Supporting Information Figure S1C).

TLR4 inhibitors, CRC and RSV, reduced PAR_2AP–induced relaxation in naive and LPS-treated rats

In order to investigate any cross-talk between PAR_2 and TLR4 signalling, aortic rings were treated with two different compounds recently identified as inhibitors of the TLR4 pathway, CRC and RSV. CRC inhibits both ligand-induced (MyD88-dependent pathway) and ligand-independent (MyD88-independent, TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway) dimerization of TLR4 (Youn *et al.*, 2006; Son *et al.*, 2008; Lubbad *et al.*, 2009), while RSV inhibits specifically the MyD88-independent, TRIF-dependent pathway (Youn *et al.*, 2005; Son *et al.*, 2008; Lubbad *et al.*, 2009). In order to verify the specificity of CRC and RSV, the maximal concentrations of either CRC (30 µM) or RSV (10 µM) were tested on ACh-induced vasodilatation. As shown in Figure 1, neither compound affected ACh –induced vasodilatation.

Both CRC (3, 10, 30 µM) and RSV (1, 3, 10 µM) significantly and in a concentration-dependent manner reduced PAR_2AP–induced vasorelaxation in naïve rats (Figures 2A, 3A). Similarly, in LPS-treated rats, both CRC (Figure 2B and C) and RSV (Figure 3B and C) also significantly inhibited PAR_2AP–induced vasorelaxation at the different time points tested. The inhibitory effects of CRC and RSV on PAR_2AP-induced vasorelaxation were not significantly different between aortic rings from naïve and endotoxaemic rats. Moreover, to exclude the possibility that the effect of CRC and RSV on PAR_2AP–induced vasorelaxation was caused by their antioxidant properties, we pre-treated aortic rings with SOD (300 $U \cdot mL^{-1}$) at a concentration known to act as superoxide anion scavenger. SOD did not modify the PAR_2AP–induced vasorelaxation (EC_{50} = 3.98 × 10^{-6} M and 3.80 × 10^{-6} M in the presence of vehicle and SOD, respectively, data not shown). Finally, to further confirm the specificity of CRC and RSV on TLR4 signalling, both inhibitors were tested on aortic rings harvested from $TLR4^{-/-}$ mice. As shown in Figure 4C, both inhibitors failed to inhibit PAR_2AP–induced vasodilatation, while they still significantly inhibited the vasodilatation in vessels obtained from C57BL/10ScN mice (Supporting Information Figure S3C).

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 $mg \cdot kg^{-1}$) and RSV (30 $mg \cdot kg^{-1}$) were administered before i.v. administration of PAR_2AP. Both CRC and RSV inhibited PAR_2AP-induced hypotension *in vivo* in naïve rats (Figure 5).

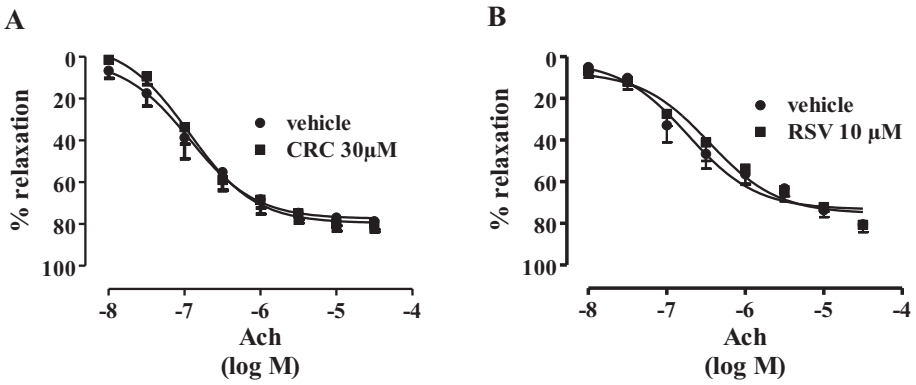

Figure 1

Pre-incubation of aortic rings with CRC and RSV did not affect ACh-induced vasodilatation. For each set of experiments, $n = 4$ rats.

Physical interaction between PAR_2 and TLR4 in naive and LPS-treated rats

To gain further insights into the molecular mechanism of PAR_2/TLR4 cross-talk an immunoprecipitation study was carried up. In homogenates of aortas obtained from both naïve and LPS-treated rats, immunoprecipitation of TLR4 receptor followed by PAR_2 immunoblot was performed. Immunoblotting of anti-TLR4 immunoprecipitates with anti-PAR_2 revealed that PAR_2 was associated with the TLR4 receptor, as a 40-kDa band consistent with PAR_2 core protein (Gruber *et al.*, 2004) was obtained (Figure 6B lane B). The complex between the PAR_2 and TLR4 receptor was also evident after *in vitro* stimulation of aortas with PAR_2AP (Figure 6B lane D). These results suggest that PAR_2 was associated with TLR4 before, as well as, after receptor activation. Similar findings have also been obtained in aortas obtained from endotoxaemic animals (Figure 6B lane E–H). To evaluate the specificity of the 40-kDa band, cell lysates of aortas stimulated or not with PAR_2AP from control and endotoxaemic rats were incubated with the corresponding normal rabbit serum instead of anti-TLR-4 antibody (Figure 6B lanes A, C, E, and G). Western blot analysis performed on the supernatants obtained from anti-TLR4 immunoprecitates revealed the presence of PAR_2 receptors exclusively in aortas harvested from LPS-treated rats (Figure 6C).

PAR_2 expression is reduced in $TLR4^{-/-}$ mice

In order to confirm the cooperation between PAR_2 and TLR4 receptors, we performed a functional study on $TLR4^{-/-}$ mice. In isolated aortic rings from these mice both ACh-induced vasodilatation and the endothelium-independent dilator agent SNP (sodium nitroprusside), induced a similar sized response in $TLR4^{-/-}$ and wild-type mice (Figure 4A and B). RT-PCR analysis revealed a significant reduction in PAR_2 expression in aortas of $TLR4^{-/-}$ mice compared with those from wild-type mice (Figure 7A). This finding was confirmed in the functional study where, PAR_2AP–induced vasorelaxation was significantly reduced in aortic rings harvested from $TLR4^{-/-}$ mice compared with wild-type mice (Figure 7C). Western blot analysis for PAR_2 did not reveal any significant difference between $TLR4^{-/-}$ and wild-type mice (Figure 7B).

Discussion

We have previously shown that PAR_2 is involved in LPS-induced hypotension; however, we did not elucidate the mechanism underlying this involvement of PAR_2 in septic shock (Cicala *et al.*, 1999). At the time, it was not known that the action of LPS mainly produced through an effect on the TLR4 receptor. Following recent evidence suggesting a possible connection between PARs and TLR signalling (Moretti *et al.*, 2008; Rallabhandi *et al.*, 2008; Uehara *et al.*, 2008), here we sought to investigate any possible cross-talk between the PAR_2 and TLR4 in vascular tissues. To pursue this, we used two different approaches: the first *ex vivo*, pharmacological modulation of isolated aortic rings obtained from naïve and endotoxaemic rats. The second *in vivo*, using PAR_2AP-induced hypotension in anaesthetized rats in order to validate the data obtained *ex vivo*. The rationale for using aorta from LPS-treated rats relies on the finding that LPS injection induces an increase in PAR_2 expression in vascular tissue.

Firstly, we evaluated whether RSV and CRC, two recently recognized TLR4 antagonists (Youn *et al.*, 2006; Son *et al.*, 2008; Lubbad *et al.*, 2009), could reduce PAR_2AP–induced vasorelaxation. In aortas from both control and LPS-treated rats, both of these antagonists significantly inhibited the PAR_2AP–induced vasodilatation without affecting NO release. The specificity of these effects of CRC and RSV on TLR4 receptor were confirmed by using aortic rings from $TLR4^{-/-}$ mice; the two antagonists had no effect on PAR_2AP-induced vasodilatation in aortic rings obtained from $TLR4^{-/-}$, while both retained their inhibitory activity on aortas obtained from C57BL/10ScN mice. The finding that the TLR4 antagonists had similar effects in aortic rings from either control or LPS-treated rats suggests that a constitutive functional interaction may exist between PAR_2 and TLR4. This latter result together with the finding that PAR_2, but not TLR4, expression is increased in aortic tissue obtained from rats 4 and 8 h after they had been injected with LPS, indicate that the PAR_2 overexpressed following LPS treatment is not physically associated with TLR4. This LPS-induced increase in the expression of the PAR_2, measured by quantitative RT-PCR, was also observed in the aortas and kidneys harvested from $TLR4^{-/-}$

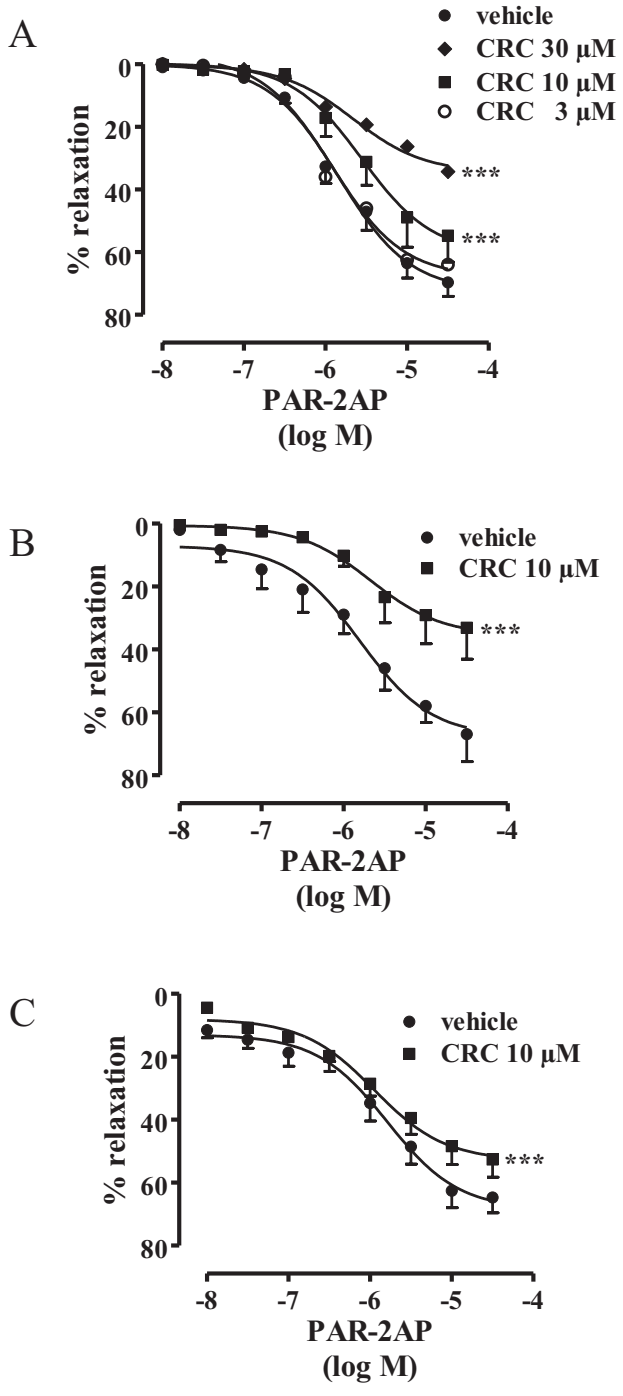

Figure 2

(A) PAR_2AP–induced vasorelaxation was significantly and concentration-dependently inhibited by CRC in aortic rings harvested from control rats (*** $P < 0.001$ versus vehicle; two-way ANOVA). CRC (10 µM) pretreatment also inhibited PAR_2AP-induced vasorelaxation in aortic rings harvested from LPS-treated rats at (B) 4 h (*** = $P < 0.001$ vs. vehicle) and (C) 8 h (*** = $P < 0.001$ vs. vehicle; for each set of experiments, $n = 5$ rats.

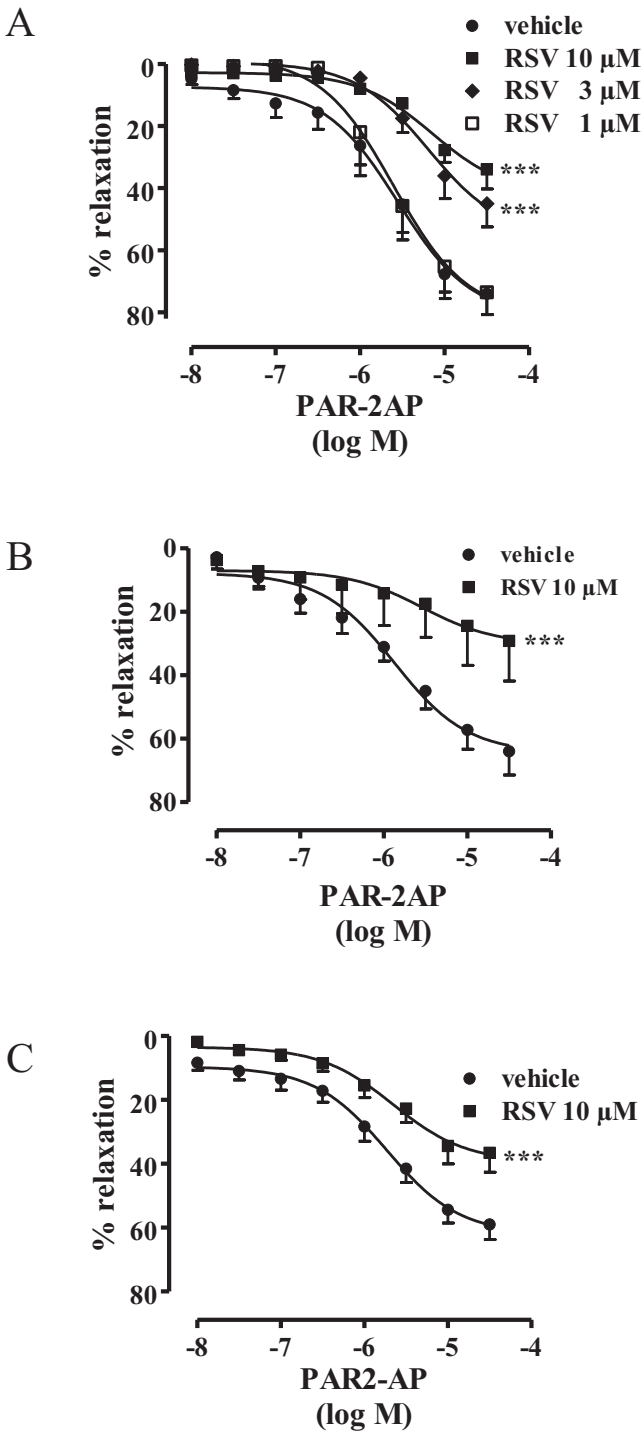

Figure 3

(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) pretreatment also inhibited PAR_2AP vasorelaxation in aortic rings harvested from LPS-treated rats at (B) 4 h (*** = $P < 0.001$ vs. vehicle) and (C) 8 h (*** = $P < 0.001$ vs. vehicle; for each set of experiments $n = 5$ rats).

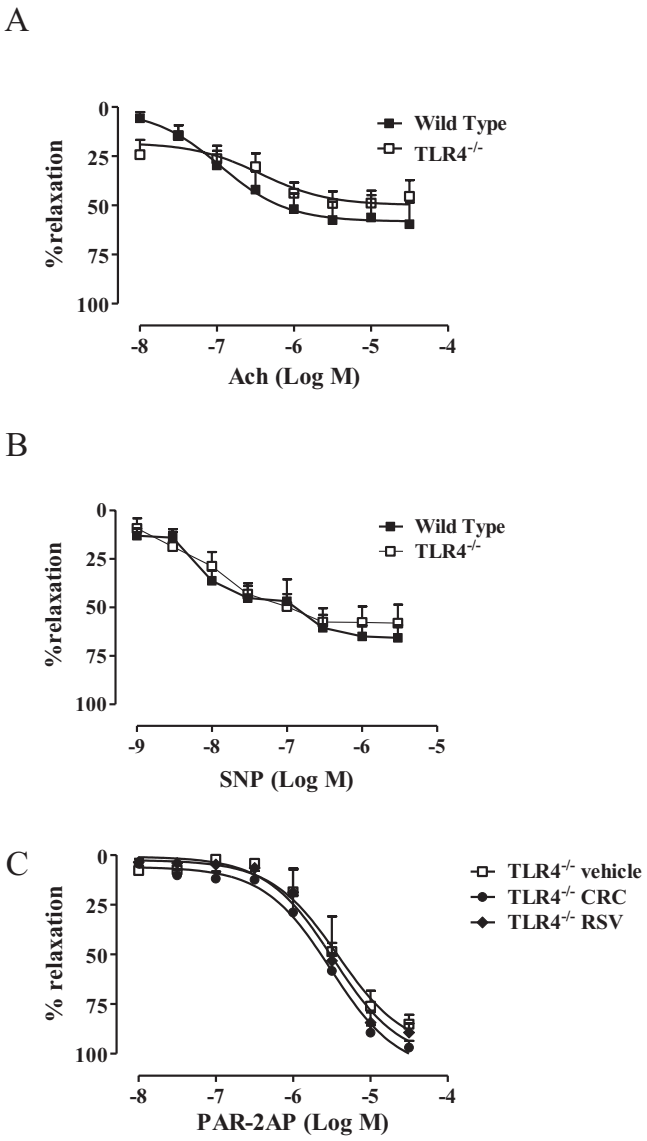

Figure 4

ACh-induced vasodilatation (10 nM–30 μM) (A), as well as SNP-induced vasodilatation (1 nM–3 μM) (B), were not significantly different in isolated aortic rings harvested from $TLR4^{-/-}$ mice compared with wild-type littermates, (C) incubation with CRC or RSV did not affect PAR_2AP-induced vasodilatation in aortic rings harvested from $TLR4^{-/-}$ mice. $n = 6$ for each group of animals.

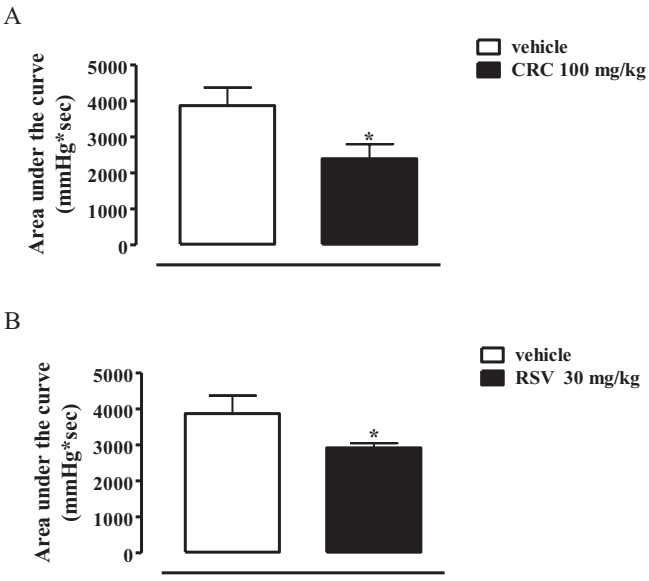

Figure 5

PAR_2AP-induced hypotension (0.3 mg·kg^{-1} i.v.) was significantly reduced in control rats treated either with (A) CRC or (B) RSV (*$P < 0.05$; one way ANOVA, $n = 4$ for each treatment). Values were expressed as area under the curve (mm^2).

mice (Supporting Information Figure S2) confirming that LPS-induced PAR_2 overexpression is independent of TLR4 expression not only in the vasculature but also in other tissues. Hence, the results of this immunoprecipitation study suggest that PAR_2 can interact with TLR4 in normal conditions and that LPS treatment does not increase this interaction. These data are in line with our hypothesis that the PAR_2/TLR4 association is constitutive in vascular tissue and LPS-induced PAR_2 overexpression does not involve a further increase in the interaction of PAR_2 with TLR4. However, as shown in Supporting Information Figure S3, the PAR_2 monoclonal antibody SAM11 appears to cross-react with an unknown target in proteins isolated from PAR_2 knockout mice, as also shown by others (Kagota *et al.*, 2011). In addition, the same applies to the polyclonal TLR4 antibody [TLR4 (M300) sc-30002] used in this study. Therefore the antibody reagents available are not yet reliable enough to clearly establish this interaction in intact tissues.

In order to test whether the above findings are also relevant *in vivo*, we treated naïve rats with RSV or CRC and then induced hypotension by a bolus injection of PAR_2AP. CRC and RSV significantly reduced PAR_2AP–induced hypotension; the dose of these antagonist used is known to specifically inhibit TLR4, as opposed to higher doses (Bengmark, 2006; Silan, 2008). These data suggest that TLR4 signalling is involved in PAR_2 activation. The fact that RSV, the MyD88-independent TRIF-dependent pathway inhibitor, was a more potent inhibitor than CRC in both the *ex vivo* and *in vivo* experiments suggests that PAR_2 activation might involve this specific TLR4 signalling pathway. This mechanism has previously been proposed by Rallabhandi and colleagues, from results obtained using transiently PAR_2 transfected HEK293T cells (Rallabhandi *et al.*, 2008). Thus, our data obtained *in vivo* confirm the results obtained from our *ex vivo* experiments, all indicating that PAR_2/TLR4 cross-talk is involved in the modulation of the vascular tone in physiological conditions. When a pathological stimulus occurs, such as LPS injection, there is an overexpression of PAR_2 that is no longer associated with TLR4.

The experiments carried out in $TLR4^{-/-}$ mice provide a proof of concept of this cross-talk between PAR_2 and TLR4. RT-PCR analysis showed that the aortas obtained from $TLR4^{-/-}$ mice had a reduced expression of PAR_2. Similarly, the functional study revealed a significant reduction in PAR_2AP–induced vasodilatation in aortic rings from $TLR4^{-/-}$ mice

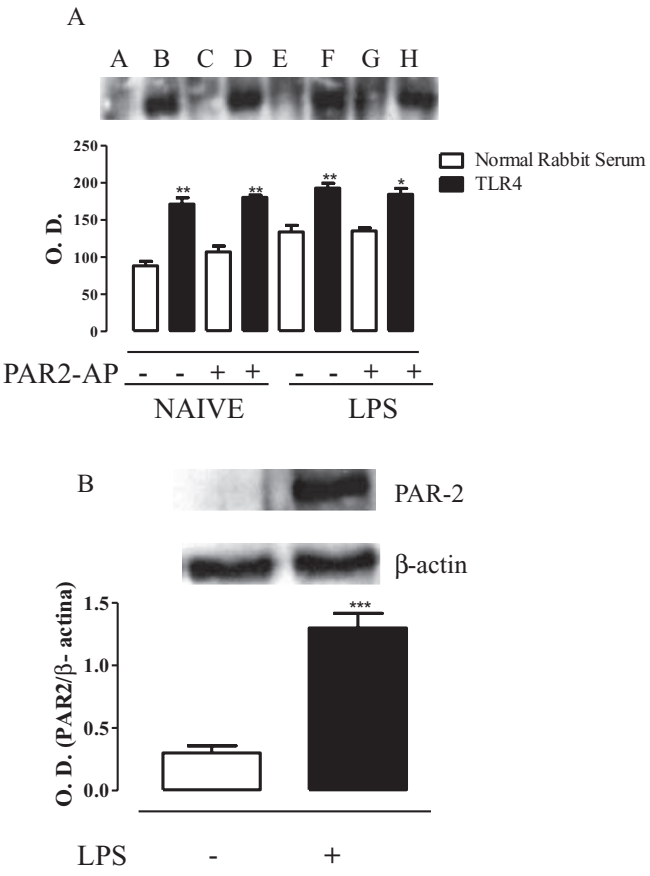

Figure 6

(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 the 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 endotoxaemic (lanes E, F, G, H) rats (n = 3 experiments). (B) Western blot analysis for PAR_2 receptor performed on supernatants of TLR4 immunoprecipitates showed the presence of PAR_2 exclusively in aortas harvested from LPS-treated rats (n = 3 experiments).

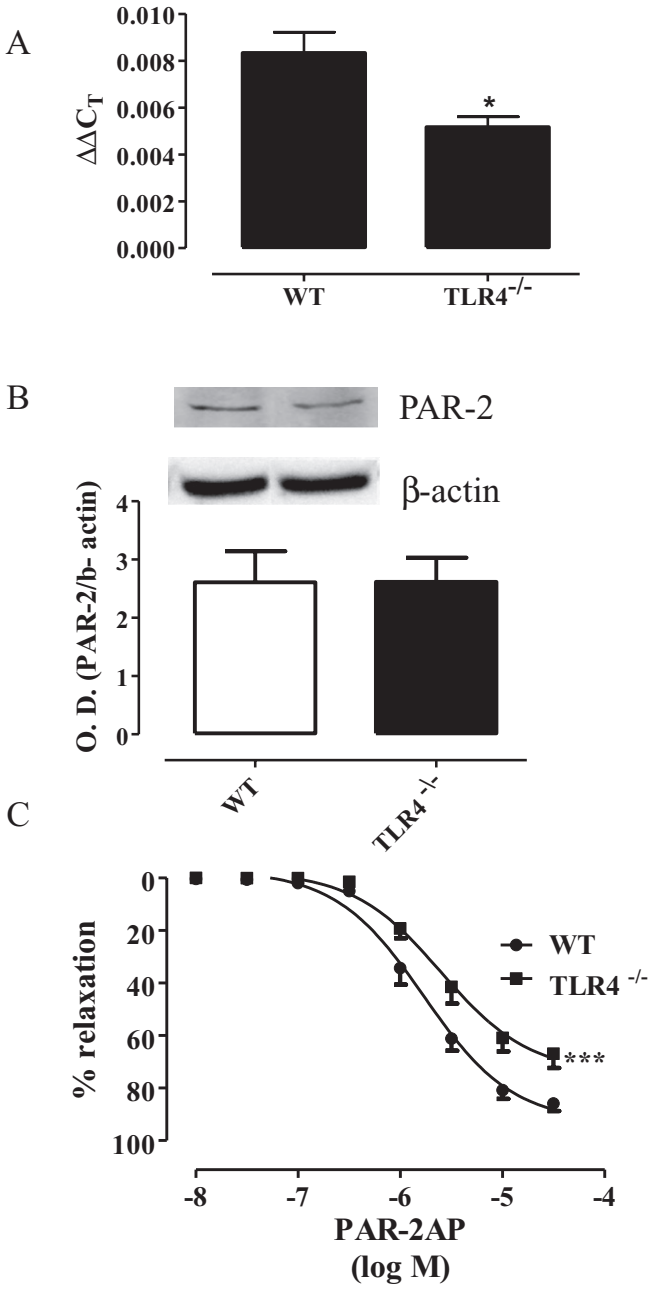

Figure 7

(A) RT-PCR analysis performed on aortas harvested from $TLR4^{-/-}$ and wild-type mice showed a reduction of PAR_2 expression in $TLR4^{-/-}$ mice (* $P < 0.05$, $n = 3$ experiments). (B) Western blot analysis of PAR_2 performed on lysates of aortas harvested from $TLR4^{-/-}$ and wild-type mice revealed no significant differences between the strains (n = 3 experiments). (C) PAR_2AP-induced vasorelaxation was significantly impaired in aortic rings harvested from $TLR4^{-/-}$ mice (***$P < 0.001$, n = 7 for each strain).

compared to wild-type mice. In order to assess the vascular reactivity of $TLR4^{-/-}$ mice, we checked both endothelium-dependent (with ACh) and endothelium-independent (with SNP) vasodilatation and showed that this was not significantly different from that of wild-type mice. These data indicate that, in the absence of TLR4, PAR_2 signalling is impaired most likely because of the lack of cooperation between PAR_2 and TLR4.

Conclusions

Our study demonstrates that in vascular tissues, PAR_2 and TLR4 cooperate in terms of molecular signalling both in physiological and pathological conditions. LPS priming causes an overexpression of PAR_2, which exceeds the TLR4 binding capacity. This finding sheds new light on the dual role of PAR_2 in physiological versus pathological conditions. Indeed, we hypothesize that when the amount of PAR_2 expressed exceeds TLR4 binding capacity, PAR_2 could elicit a detrimental effect. Conversely, when all the PAR_2 expressed are bound to TLR4 it would have a beneficial physiological effect. Our findings could explain why in some cases activa-

tion of PAR_2 has been found to induce anti-inflammatory effects (Fiorucci *et al.*, 2001; Morello *et al.*, 2005; Roviezzo *et al.*, 2005), whereas others have demonstrated that it results in pro-inflammatory effects (Fiorucci *et al.*, 2001; Ferrell *et al.*, 2003; Hyun *et al.*, 2008), and they also help to unravel the complex role played by this protease-activated receptor in cardiovascular homeostasis.

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) Western blot analysis shows a significant increase in PAR_2 expression in LPS (13.6 $U \cdot kg^{-1}$) treated rats. (B) PAR_2AP-induced vasodilatation was significantly increased in aortic rings harvested from LPS-treated rats (4 and 8 h after LPS) $^*P < 0.05$ versus saline; $^{***}P < 0.001$ versus saline; $n = 10$ for each group. (C) Western blot analysis revealed no difference in TLR4 expression in LPS-treated compared with naive rats.

Figure S2 qRT-PCR analysis performed on kidney (A) or on aorta (B) harvested from $TLR4^{-/-}$ mice demonstrates that LPS causes an upregulation of PAR_2 expression. ($^*P < 0.05$ $^{***}P < 0.001$; $n = 3$ experiments).

Figure S3 (A) Western blot analysis showing PAR_2 expression in aorta from wild-type and PAR_2 KO mice. (B) Western blot analysis showing TLR4 expression in aorta from wild-type and TLR4 KO mice. Blots are representative of 3 different experiments. (C) CRC (10 μM) and RSV (10 μm) significantly inhibited PAR_2AP-induced vasodilatation in aorta harvested from C57BL/10ScN mice ($^{***} = P < 0.001$ vs. vehicle; $n = 3$ mice for a total of 10 rings for each group).