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Arterial Thrombosis Is Accompanied by Elevated Mitogen-Activated Protein Kinase (MAPK) and Cyclooxygenase-2 (COX-2) Expression via Toll-Like Receptor 4 (TLR-4) Activation by S100A8/A9

Authors' Contribution:
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Statistical Analysis C
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Background: The aim of this study was to determine the involvement of S100A8/A9 in the development of arterial thrombosis.

Material/Methods: A total of 303 patients were enrolled in this study, with 110 having acute coronary syndrome (ACS) and 110 having coronary heart disease (CHD), and 83 subjects served as healthy blood donors. The concentrations of Toll-like receptor 4 (TLR-4), cyclooxygenase-2 (COX-2), and S100A8/A9 protein were determined in the sera of the participants and in peripheral blood mononuclear cells (PBMCs) derived from a rat carotid artery thrombosis model and in human aortic endothelial cells (HAECs). The mitogen-activated protein kinase (MAPK) inhibitor SB203580 and the TLR-4 blocker CLI-095 were used to investigate the role of the TLR-4-MAPK-COX2 signaling axis in thrombosis.

Results: The levels of COX-2, TLR-4, and S100A8/A9 in the sera of patients with ACS and CHD were significantly higher than in healthy controls ($P < 0.05$). S100A8/A9 expression was significantly correlated with TLR-4 and COX-2 in the ACS group and with TLR-4 in the CHD group. In the rat carotid thrombosis model, the expressions of TLR-4, COX-2, and p-p38 MAPK significantly increased until 14 days after thrombosis induction, whereas S100A8/A9 expression increased until day 7, but then decreased. Administration of SB203580 to rats reduced COX-2 expression in PBMCs after thrombosis induction, and incubation of HAECs with CLI-095 reduced their p-p38 MAPK and COX-2 response to S100A8/A9 stimulation.

Conclusions: S100A8/A9 is upregulated after blood vessel injury and is enhanced in combination with TLR-4 COX-2 induction via p38 MAPK activation.

MeSH Keywords: **Cyclooxygenase 2 • Inflammation Mediators • Mitogen-Activated Protein Kinases • Thrombosis • Toll-Like Receptor 4**

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Background

During the last decade much effort has been expended to find biomarkers that can be used for prognosis of the risks for vascular diseases [1]. Inflammation is an established risk factor for arterial thrombotic diseases [2,3]. S100 proteins play roles in the regulation of inflammation, and their expression is significantly increased in a variety of inflammatory conditions. Recent studies have suggested that increased serum levels of S100 proteins are associated with abnormal platelet aggregation and enlarged myocardial infarct size [4,5], while circulating serum S100A8/A9 levels were increased by cardiovascular risk factors and correlated with vulnerable plaque phenotypes and the extents of coronary and carotid atherosclerosis [6]. S100A8/A9 is also involved in vascular inflammation, response to vascular injury, and development of thrombohemorrhagic vasculitis [7]. Wang et al. (2014) showed that S100A8/A9 decreased the carotid artery occlusion time after injury in a mouse S100A9 knock-out experiment [8].

Toll-like receptor 4 (TLR-4) is a transmembrane signaling receptor that exerts its actions through p38 mitogen-activated protein kinase (MAPK) [9]. TLR-4 is involved in the development of atherosclerosis, pathological myocardial remodeling, and tissue damage in ischemic stroke [10,11]. It has been shown that TLR-4 causes vasoconstriction and thrombosis formation following endothelial injury [12] and is an essential factor in infection-related thrombosis [13,14]. COX-2 is induced by p38 MAPK [15] and is barely detectable in most tissues under physiological conditions but is upregulated in human atherosclerotic lesions [16]. Since S100A8/A9 is an endogenous ligand of TLR-4 and a previous study noted the involvement of S100A8/A9/TLR-4 signaling in various inflammatory conditions [17–20], in the current study we measured the plasma levels of S100A8/A9, TLR-4, and COX-2 in patients with acute coronary syndrome (ACS) and coronary heart disease (CHD). We also investigated the interplay of these factors in cultured endothelial cells and in a rat carotid thrombosis model to determine their usefulness as biomarkers for the diagnosis of vascular disorders.

Material and Methods

Ethics statement

Our study was performed in accordance with the Declaration of Helsinki with regard to ethical principles for research involving human subjects and was approved by the Research Ethics Committee of Rui Jin Hospital. Written informed consent was obtained from all patients.

The animal experiments were based on ethical considerations and integrity-based assumptions and the experimental protocol was approved by the Institutional Animal Ethics Committee of Rui Jin Hospital, which follows the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Patients

Patients with cardiovascular disease (CVD) admitted to the Department of Geriatrics, Rui Jin Hospital included 110 cases of acute coronary syndrome (ACS) (60 men, 50 women, age range: 60–92 years, mean age 67.02 ± 12.13 years) and 110 cases of coronary heart disease (CHD) (55 men, 55 women, age range 60–90 years, mean age 64.50 ± 8.10 years). None of the participants had a history of: (1) other diseases, including severe liver and kidney function impairment; (2) severe heart failure; (3) thyroid dysfunction; (4) systemic autoimmune disease; (5) chronic inflammation; (6) severe infection, tumor or contagion; (7) cerebral hemorrhage; (8) membranous heart disease, rheumatic heart disease, or myocarditis; (9) or treatment with heart, lung, or other organ transplantations. The plasma was separated from EDTA-anticoagulated whole-blood samples collected from the patients. Plasma samples from 83 healthy blood donors (43 men, 40 women; age range 60–90 years (mean 67.46 ± 7.71 years) were used as normal references.

Peripheral blood sampling, processing, and detection

Peripheral blood samples were obtained following an overnight fast and centrifuged (3000 rpm) at room temperature. Plasma aliquots were stored at -80°C until assayed. Plasma S100A8/A9, TLR-4, and COX-2 levels were measured by ELISA (COX-2 detection kit, Cell Signaling Technology, M; TLR-4 detection kit, Abnova, Taipei, China; S100A8/A9 detection kit, Immundiagnostik AG, Bensheim, Germany).

Culture and stimulation of arterial endothelial cells by S100A8/A9

Human aortic endothelial cells (HAECs) (ScienCell, Carlsbad, USA) were maintained in DMEM/F12 medium containing 8% FBS and penicillin/streptomycin for 12 h. The cells were divided into 2 groups. One group was treated with S100A8/A9 (Hycult Biotech, Frontstraat, Netherlands) at a concentration of $4.0 \mu\text{g}/\text{mL}$ for 2 h. Cells in the other group were incubated with the TLR-4 blocker cli-095 (InvivoGen, Carlsbad, USA) for 30 min prior to S100A8/A9 stimulation. The cells were collected and analyzed by Western blotting.

Rat carotid thrombosis model

Sprague-Dawley rats (200~250 g) were obtained from the Shanghai Institute of Animals, Chinese Academy of Sciences (Qualified number: SYXK (Shanghai), 2013-0062). Forty rats were randomly divided into treatment (T, n=20) and control (C, n=20) groups. The groups were further divided into 5 subgroups: 3-h groups (T/C 3 h, n=4 each), 1-day groups (T/C 1d, n=4 each), 3-day groups (T/C 3d, n=4 each), 7-day groups (T/C 7 d, n=4 each), and 14-day groups (T/C 14 d, n=4 each). After routine feeding for 1 week, rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg) and the left common carotid artery was exposed by cervical incision. After separation from the adherent tissue, a 3×1.8 cm PE plastic film was placed under the carotid artery for protection of the surrounding tissue. Then, a 1-cm long filter paper saturated with 20% FeCl₃ solution (anhydrous FeCl₃, Sigma Aldrich, St. Louis, USA) was placed under the PE plastic film and wrapped around the left common carotid artery for 15 min. After removal of the filter paper and the PE plastic film, the remaining FeCl₃ solution was washed away with saline solution, followed by suturing of the incision [21]. The control group was treated with normal saline instead of FeCl₃ solution. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg) at 3 h, 1 day, 3 days, 7 days, or 14 days after the procedure, after which 2 ml of blood was collected from the abdominal aorta for serum and PBMC analyses as described below and after cervical dislocation-injured vessel segments were excised and fixed with 4% paraformaldehyde in PBS buffer.

Extraction of rat PBMCs

Ficoll-Hypaque density gradient centrifugation was utilized for extraction of PBMC. Briefly, 2 mL of whole blood was collected from the rats of the rat carotid thrombosis model and anticoagulant-treated with EDTA. Following a 1: 1 dilution with PBS solution, the suspension was added on top of a Ficoll-Hypaque PREMIUM 1.084 separation solution (GE Healthcare, Uppsala, Sweden) at a 1: 1 volume. After horizontal centrifugation (2000 rpm) for 20 min, PBMCs separated to the second layer of the separation solution. PBMCs were collected and added to 1 mL of RIPA lysis solution together with 20 mL of the protease inhibitor PMSF, followed by a 10-min incubation on ice. After a 10-min centrifugation (12 000×g) at 4°C, the supernatants were separated and the protein concentrations in the samples were determined using the BCA method.

Hematoxylin and eosin (H&E) staining and immunohistochemistry

The rat carotid artery samples were embedded in paraffin and sliced into 5-µm sections. The sections were stained with H&E and used for routine histological examination. Mononuclear

macrophages were detected by immunohistochemical staining using a goat anti-rat CD68 polyclonal antibody (Cell Signaling Technology, Danvers, USA) as the primary antibody and a horseradish peroxidase (HRP) labeled donkey anti-goat antibody as the secondary antibody (Abcam, Cambridge, USA).

Western blotting

Rat sera and denatured PBMCs protein extract were subjected to SDS-PAGE and then transferred to PVDF membranes, which were then incubated with primary antibodies against TLR-4, p38 MAPK/p-p38 MAPK, and COX-2 (Cell Signaling Technology, Danvers, USA) overnight at 4°C. The HRP-labeled goat anti-rabbit polyclonal antibody (Cell Signaling Technology, Danvers, USA) was used to detect bound primary antibody and a chemiluminescent substrate (PerkinElmer, MA, USA) was utilized to visualize the signal. Densitometry of blots was performed with Image J analysis software (NIH, Bethesda, USA).

Inhibition of MAPK-dependent signaling pathway in a rat thrombosis model

To assess the contribution of inflammation to thrombosis formation *in vivo*, the MAPK-dependent signaling pathway was inhibited by SB203580 (10 mg/kg, intraperitoneal injection 2 h prior to surgery and 1 mg/kg once daily for 7 days after surgery in 4 rats undergoing FeCl₃-induced vessel injuries (intervention group, SB+), while 4 other rats with carotid artery thrombosis formation but no MAPK inhibitor treatment were used as the control group (SB-). All rats were sacrificed 7 days post-surgery and PBMCs were collected using Ficoll-Hypaque density gradient centrifugation.

Statistical analysis

All statistical analyses were performed using SPSS for Windows (Version 16.0. Chicago, SPSS Inc.). One-way ANOVA was adopted if homoscedasticity was assumed; otherwise, data were either analyzed after transformation or a Kruskal-Wallis test were used. Correlation coefficients were calculated using Pearson's statistic. All tests were 2-sided and *P*<0.05 was considered to be statistically significant.

Results

Patients' baseline information

There were no significant differences in sex, total cholesterol, and high-density lipoprotein among the ACS, CHD, and normal control groups. The serum concentrations of biomarkers representing myocardial impairment, such as CK-MB, myoglobin, creatine kinase, and lactate dehydrogenase, as well as cTnI

Table 1. The baseline information of control, CHD and ACS groups.

		Control group (n=83)	CHD group (n=110)	ACS group (n=110)	P values		
					CHD vs. control	ACS vs. control	ACS vs. CHD
Gender	Male	43 (53.8%)	55 (50.0%)	60 (54.5%)	0.459	0.408	0.295
	Female	40 (46.2%)	55 (50.0%)	50 (45.5%)			
Age (years)		67.46±7.71	64.5±8.10	67.02±12.13	0.0111	0.0714	0.7727
White blood cell count (×10 ⁹ /L)		5.41±1.20	5.43±1.28	8.57±2.61	0.9122	<0.0001	<0.0001
Neutrophils (%)		56.20±8.26	59.72±9.51	71.61±8.37	0.0077	<0.0001	<0.0001
Triglycerides (mmol/L)		1.20±0.40	1.32±1.22	1.47±0.65	0.3901	0.2563	0.001
Total cholesterol (mmol/L)		4.37±0.94	4.23±1.02	4.39±1.19	0.3302	0.2855	0.8997
High-density lipoprotein (mmol/L)		1.35±0.28	1.35±0.18	1.35±0.35	1.0000	1.000	1.000
Low-density lipoprotein (mmol/L)		2.70±0.78	2.47±0.93	2.80±0.98	0.0702	0.0111	0.4455
Lactate dehydrogenase (U/L)		103.26±20.37	125.77±40.26	379.36±425.03	<0.0001	<0.0001	<0.0001
Creatine kinase (U/L)		57.17±34.72	95.59±56.84	897.45±96.40	<0.0001	<0.0001	<0.0001
CK-MB (ng/mL)		0.69±0.08	1.54±0.95	48.89±86.01	<0.0001	<0.0001	<0.0001
Myoglobin (ng/mL)		23.45±9.25	34.26±11.70	78.08±36.20	<0.0001	<0.0001	<0.0001
Troponin I (cTnI) (ng/mL)		0.014±0.01	0.016±0.01	22.33±33.22	0.1706	<0.0001	<0.0001
Aspartate aminotransferase (IU/L)		21.86±4.67	28.74±36.11	96.25±145.21	0.0863	<0.0001	<0.0001

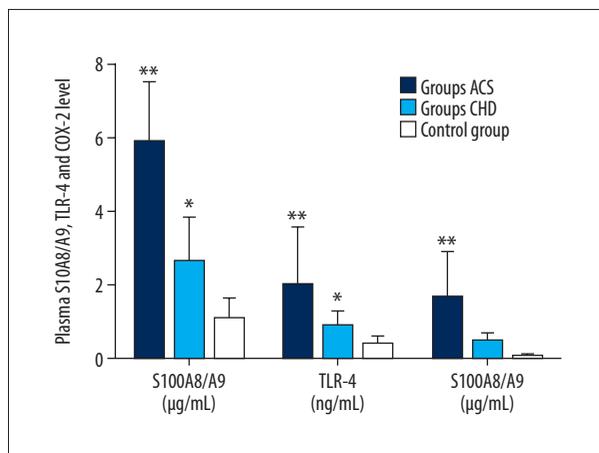


Figure 1. Blood plasma concentrations of S100A8/A9, TLR-4, and COX-2 in ACS (N=110), CHD (N=110), and healthy participants (N=83). * $P < 0.05$ and ** $P < 0.01$ compared to the control.

and aspartate aminotransferase, were most enhanced in the ACS group ($P < 0.001$) (Table 1).

Both ACS and CHD groups had significantly higher serum levels of S100A8/A9, TLR-4, and COX-2 compared to the controls ($P < 0.05$). However, elevations of S100A8/A9, TLR-4, and

COX-2 serum levels in ACS patients were more prominent than in CHD cases (Figure 1).

Serum S100A8/A9 levels were positively correlated with TLR-4 ($r = 0.754$, $P = 0.022$) and COX-2 levels ($r = 0.602$, $P = 0.036$) in the ACS group and with TLR-4 ($r = 0.586$, $P = 0.045$) in the CHD group. Similarly, the TLR-4 levels were positively correlated with COX-2 in the ACS ($r = 0.831$, $P = 0.020$) and in the CHD ($r = 0.525$, $P = 0.038$) groups. There was no obvious correlation between S100A8/A9 and COX-2 levels in the CHD patients ($r = 0.078$, $P = 0.171$) (Table 2).

Vascular structures and local inflammatory cell levels change after arterial thrombosis in a rat model

The vessel was injured upon FeCl₃ treatment (Figure 2A) and the vascular lumen was filled with dark red thrombus tissue (Figure 2Aa, 2Ba) but not in controls (Figure 2Ab, 2Bb). The tissue included platelets and infiltrated inflammatory cells, and CD68-positive mononuclear macrophages also were found at the site of injury (2C), indicating gradual recruitment of mononuclear macrophages following vascular thrombus formation over time (Figure 2C 3 h–14 d). The artery remained completely patent following saline treatment without inflammation.

Table 2. Correlations between S100A8/A9, TLR-4 and COX-2 levels in the ACS and CHD groups.

		S100A8/A9		TLR-4
		TLR-4	COX-2	COX-2
ACS	r	0.754	0.602	0.831
	P	0.022	0.036	0.020
CHD	r	0.586	0.078	0.525
	P	0.045	0.171	0.038

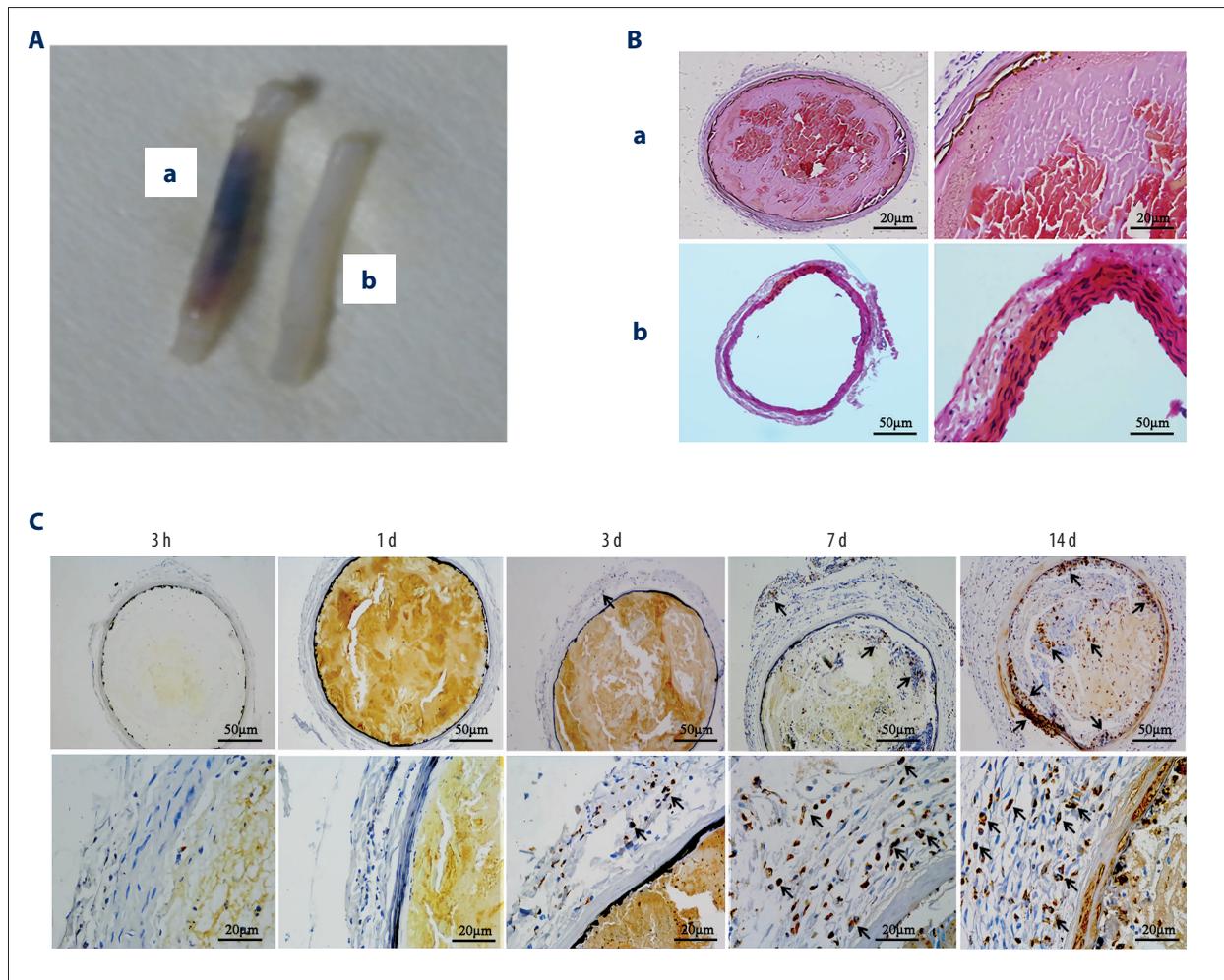


Figure 2. Changes of vascular structure and the level of local inflammatory cells following arterial thrombosis. (A) Representative view of dissected blood vessels, (a) experimental group (FeCl₃ treated n=4), (b) control group (normal saline treated, n=4); (B) HE staining results of vascular cross-sections, (a) blood vessels of the experimental group (n=4, FeCl₃ treated), (b) blood vessels of the control group (n=4, normal saline treated); left side ×100 and right side ×400 magnification. (C) CD68-labeled mononuclear macrophages are shown with brown dye (arrows) at 3 h and days 1–14 after thrombosis induction. The mononuclear macrophages appeared from day 3 and gradually migrated into the wall and lumen of locally damaged blood vessels until day 14; upper panel ×100, lower panel ×400 magnification.

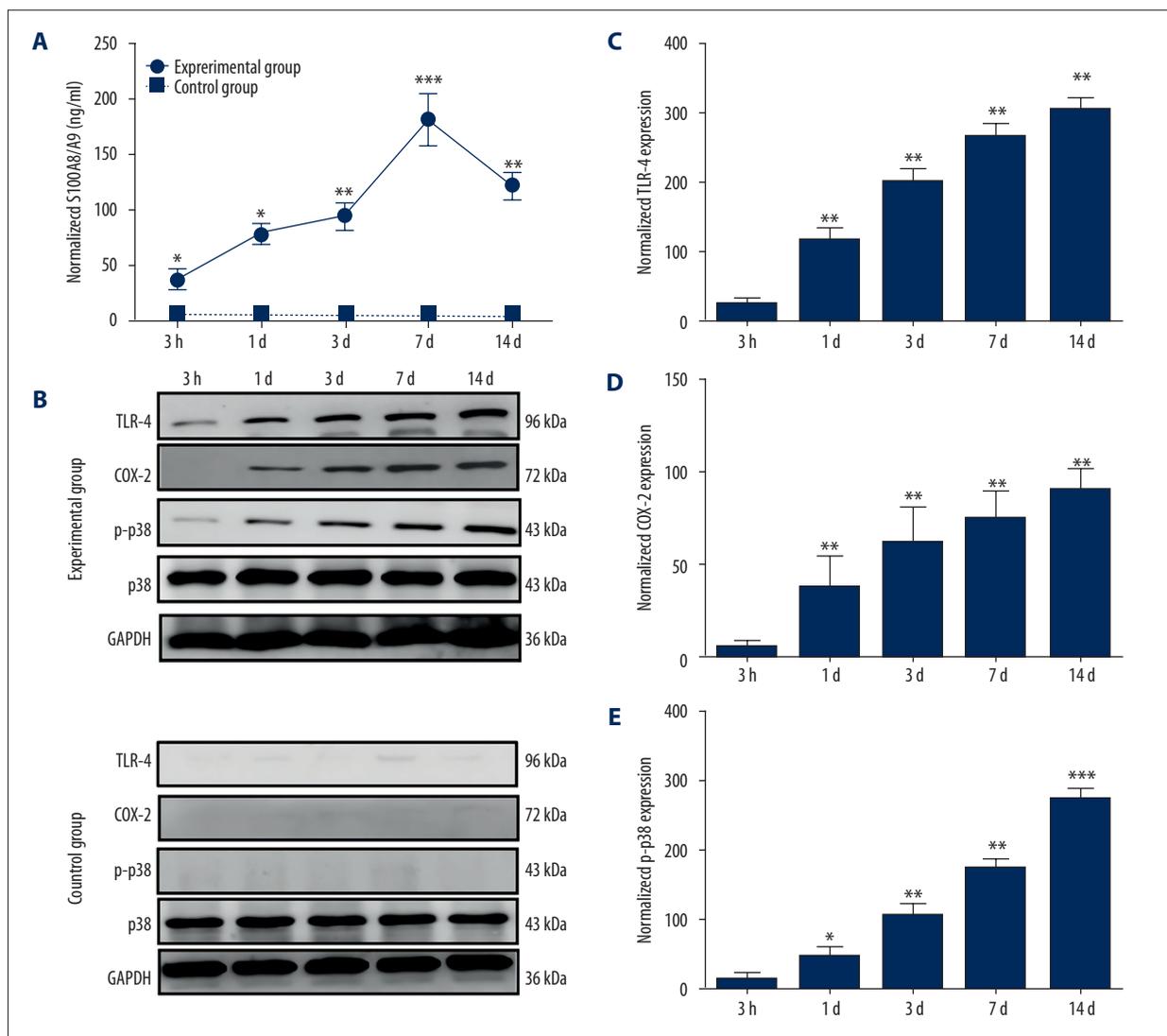


Figure 3. Western blot results of S100A8/A9, TLR-4, p-p38 MAPK, and COX-2 protein levels in rat sera and PBMCs of the rat thrombosis model. (A) Peripheral rat serum S100A8/A9 concentrations; * $P < 0.05$ and ** $P < 0.01$ and *** $P < 0.001$ compared to saline-treated rats. (B) Representative Western blot results of TLR4, COX-2, p-p38, and p38 expressions in isolated PBMCs from experimental and control group rats; Charts of (C) Normalized TLR-4 (D) COX-2 and (E) p-p38 MAPK expressions in PBMCs of the experimental rat group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to 3 h data. All experiments were done 3 times independently. 3 h – 3 h after thrombosis induction; 1 d–14 d – 1–14 days after thrombosis induction.

Serum S100A8/A9 levels and TLR-4, p-p38 MAPK, and COX-2 expression of PBMCs in the rat carotid thrombosis model

Rat serum S100A8/A9 levels rose gradually after carotid artery thrombosis formation and reached a peak on the 7th day and decreased thereafter. There was no change in S100A8/A9 levels in the control group over the same time course (Figure 3A).

After rat carotid artery injury and thrombi formation, TLR-4, p-p38 MAPK, and COX-2 expression increased gradually (Figure 3B) and the relative expression results after

normalization showed that, when compared with the 3-h group, TLR-4, COX-2, and p-p38 MAPK levels in the other groups were significantly higher (Figure 3C–3E).

Correlation between TLR-4, P-p38 MAPK, and COX-2 levels in the rat thrombosis model

In rats with carotid thrombosis formation, peripheral serum S100A8/A9 levels and TLR-4, P-p38 MAPK, and COX-2 levels in the PBMCs isolated from rats increased with various degrees of correlation. To further clarify the relationship between the MAPK inflammatory pathways and the COX-2 expression level,

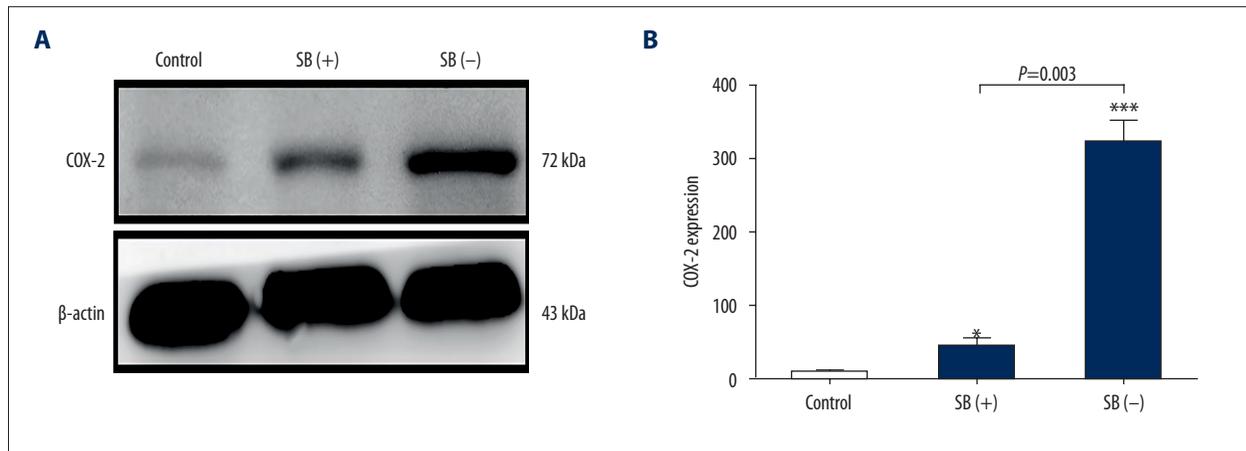


Figure 4. Influence of blockade of the MAPK pathway by SB203580 on COX-2 expression. **(A)** Representative Western blot analysis of COX-2 expression level after MAPK pathway blockade. **(B)** Charts of relative expression levels of COX-2 after MAPK pathway blockade. All experiments were done 3 times independently. SB (+) – treated with the p38 MAPK inhibitor SB203580; SB (-) – no treatment; * $P<0.05$ and *** $P<0.001$ compared to the control.

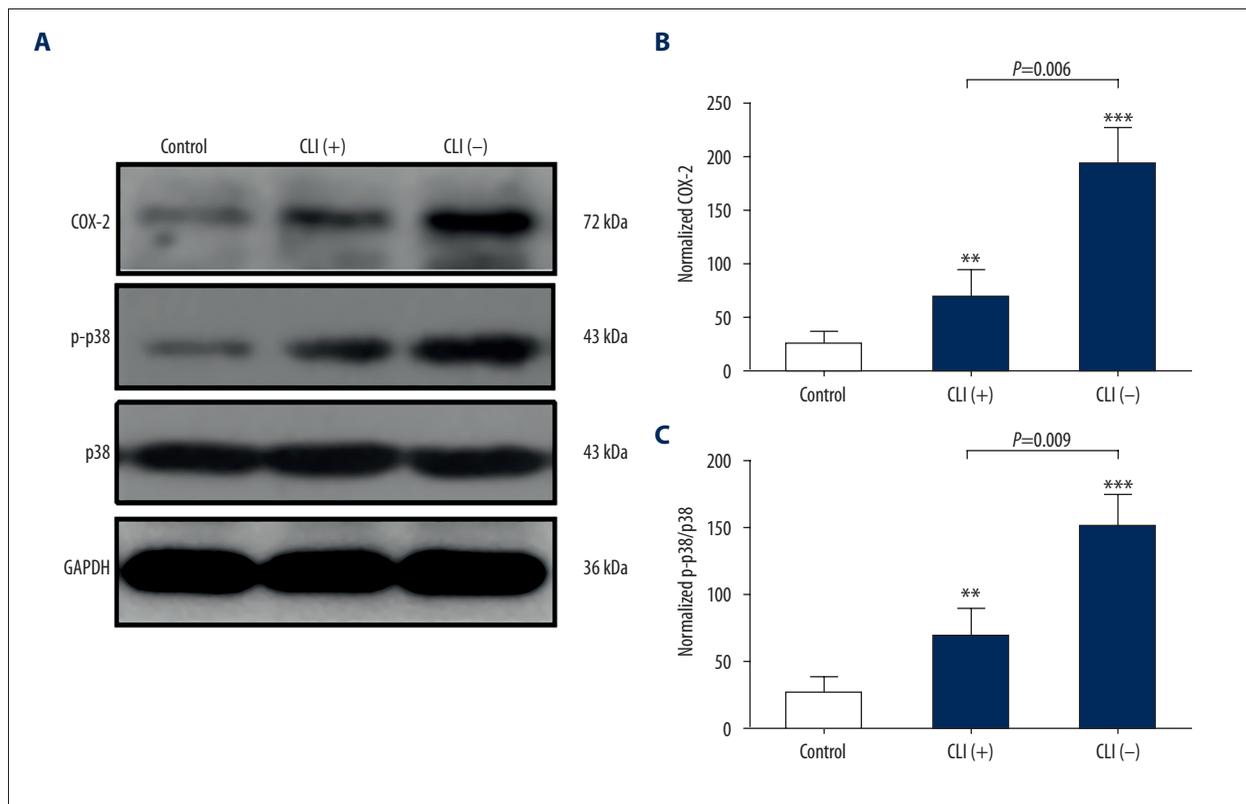


Figure 5. Changes in p-p38 MAPK and COX-2 expressions in artery endothelial cells following S100A8/A9 stimulation. **(A)** Western blot analysis of p-p38 MAPK and COX-2 expressions in artery endothelial cells after S100A8/A9 stimulus. **(B)** Normalized expression COX-2 and **(C)** p-p38 MAPK in artery endothelial cells after S100A8/A9 stimulus. All experiments were done 3 times independently. CLI (+) – TLR-4 blocked by CLI-095, CLI (-) – no treatment. ** $P<0.01$ and *** $P<0.001$ compared to the control.

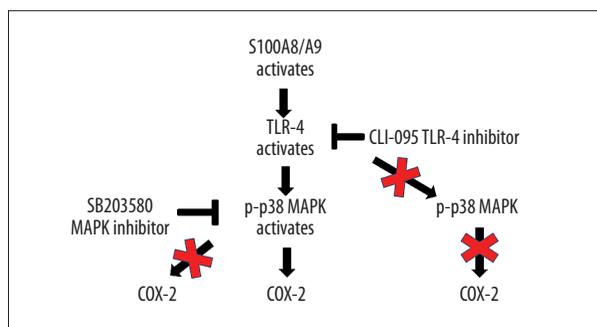


Figure 6. Scheme of signal transduction in the process of thrombosis and the effects of CLI-095, TLR-4, and SB203580 p-MAPK inhibitors. Inhibition of TLR-4 leads to reduced p-p38 MAPK and in turn reduced COX-2 expression, whereas MAPK inhibition only reduced COX-2 expression.

the MAPK pathway was blocked with SB203580. As a result, 7 days after thrombosis induction the expression of COX-2 was essentially reduced in SB203580 treated rats (SB+) compared to the rats without SB203580 application (SB-) and almost undetectable in the control rats without thrombosis (Figure 4), indicating that p38 MAPK activity is upregulating COX-2 expression after thrombosis formation.

TLR-4, p-p38 MAPK, and COX-2 levels in arterial endothelial cells following S100A8/A9 stimulation

After being treated with the TLR-4 blocker CLI-095 (CLI (+)), an exogenous S100A8/A9 stimulus was unable to promote elevations in p-p38, MAPK, or COX-2 protein expression in the CLI (+) group to the same extent as in the CLI (-) group (Figure 5A). The HAECs in the CLI (-) group that did not undergo TLR-4 blocking treatment prior to S100A8/A9 stimulation showed maximum elevated levels of p-p38 MAPK and COX-2 as compared to the CLI (+) group (Figure 5B, 5C).

The data suggested that S100A8/A9 in combination with TLR-4 activated p38 MAPK signaling pathways, which in turn induced COX-2 expression (Figure 6).

Discussion

Inflammation is a response to tissue disruption and plays a pivotal role in arterial embolism [22–24]. There is continuous recruitment of inflammatory cells at the *loci* of thrombosis formation, accompanied by elevated levels of inflammatory markers. The unregulated expression of inflammatory factors produces more damage to the tissue, resulting in cell degeneration and necrosis. The exacerbated local environment thus further boosts the formation of thrombi via a vicious inflammation/thrombosis cycle [25].

S100A8/A9 exists in the cytoplasm of neutrophils and within the cell membrane of mononuclear cells. It is secreted by activated neutrophils and monocytes or released by the rupture of dead cells. S100A8/A9 is highly expressed in a variety of inflammatory diseases such as ulcerative colitis and rheumatoid arthritis, and is used to establish the stages and prognosis of these diseases [26]. It has been suggested that S100A8/A9 can be used as a novel biomarker of cardiovascular disease, along with neutrophils count, smoking, BMI, and low-density lipoprotein serum concentrations [5]. The inflammation reactions were clearly more active in patients with ACS and CHD in our study, as suggested by increased WBC and neutrophils in the circulation. In agreement with previous studies, we demonstrated that S100A8/A9 serum levels in patients with ACS and CHD were significantly enhanced compared to healthy controls [27,28] and in accordance with a study of Schiopu and Cotoi (2013), the highest levels of serum S100A8/A9 concentrations were detected in ACS patients, in which the number of neutrophils were highest [6]. In addition, S100A8/A9 was elevated in plasma of patients upon injury transcatheter aortic valve replacement, generating a thromboinflammatory state that contributes to the frequent cardiovascular events observed in these patients [29]. Recent *in vivo* studies using a FeCl₃-induced rat carotid artery thrombosis model to corroborate our clinical findings showed increased serum S100A8/A9 levels, which is also in line with other previous studies [30–32]. TLR-4 levels in local vessels increased significantly when the artery plaque was unstable; this receptor plays an important role in atherosclerosis and pathological myocardial remodeling and can predict the occurrence of acute cardiovascular events [10,33]. Our results showed that TLR-4 levels were significantly higher in ACS and CHD patients and based on our rat thrombosis model data, we suggest that increased TLR-4 expression might be an early and long-lasting event in thrombosis formation, which is consistent with the hypothesis that enhanced TLR expression indicates inflammation and contributes to the development of vascular diseases [34].

Peripheral blood COX-2 levels in patients with CHD, and more prominently ACS patients, were significantly higher than in control subjects. COX-2 expression increased almost immediately after endothelial injury and inflammation remained at high levels in our rat model. It is possible, following arterial injury and with the numbers of inflammatory cells recruited to the local damaged blood vessels gradually increasing over time, that as a result, COX-2 is overexpressed via S100A8/A9-TLR-4 activity since we found that peripheral blood S100A8/A9, TLR-4, and COX-2 levels concomitantly significantly increased in patients with vascular diseases. It has been shown that S100A8/A9 can activate neutrophils through the MAPK pathway and lead to its degranulation [35]. Our *in vivo* studies demonstrated that S100A8/A9 might also upregulate COX-2 protein levels through activation of the p-p38 MAPK signaling

pathway, but S100A8/A9 may have been mainly involved in the early inflammatory reactions and did not contribute to sustained elevation of COX-2, as a reduction of its expression occurred from the 7th day after artery damage in our rat model.

Conclusions

Peripheral blood S100A8/A9, TLR-4, and COX-2 levels were associated with ACS and also with CHD. While combined TLR-4,

S100A8/A9, and p38 MAPK signaling pathways regulate COX-2 expression, S100A8/A9 might be mainly involved in the early process of inflammation.

Conflict of interest

None.

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