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ORIGINAL ARTICLE

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Molecular mechanism whereby paraoxonase-2 regulates coagulation activation through endothelial tissue factor in rat haemorrhagic shock model

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Abstract

We investigated the molecular mechanism of paraoxonase-2 (PON-2) in regulating blood coagulation activation in rats with haemorrhagic shock through endothelial tissue factor (TF). Thirty adult Sprague Dawley rats were randomly divided into three groups: healthy control group (group A), the haemorrhagic shock PON-2 treatment group (group B), and the haemorrhagic shock group (group C). After the model was established, blood was withdrawn from the inferior vena cava of all rats. The difference in plasma thrombomodulin (TM) levels of the three groups was determined by Western blotting. The expression of transcription factors Egr-1 and Sp1 was detected by Western blotting assays. reverse transcription-polymerase chain Reaction (RT-PCR) was used to determine the mRNA expression of t-PA, PAI-1, TM, and PON-2 in the serum of three groups of rats. Endothelial TF was measured by enzyme linked immunosorbent assay (ELISA), and coagulation assay was used to detect the activity of coagulation factor VIII. Histopathological examination of the arteries of the rats was performed. The molecular mechanism of PON-2 in regulating blood coagulation activation in haemorrhagic shock model rats by endothelial tissue factor was analysed. The expression of thrombin was determined by electrophoresis. Compared with the healthy control group, the expression of TM in groups B and C decreased, both 188.64 ± 12.47 and 137.48 ± 9.72, respectively, with a significant difference. The mRNA expression of TM and PON was determined by RT-PCR. The mRNA expression of TM and PON in group B was 0.97 ± 0.07 and 1.14 ± 0.09, compared with the control group, and the mRNA expression of TM and PON in group C was 0.86 ± 0.38 and 1.12 ± 0.41, both of which increased, and there were significant differences. By measuring the expression of endothelial TF, the expression of TF in groups B and C was elevated to 12.69 ± 1.07 and 11.59 ± 0.87, with significant differences. The enzyme activities of PON-2 in groups B and C, which were 110.34 ± 14.37 and 52.37 ± 8.06, respectively, were increased compared with the healthy control group and there were significant differences. PON-2 regulates the activation of coagulation in rats with haemorrhagic shock

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by regulating the expression of endothelial tissue-related genes such as plasma TM and endothelial TF under hypoxic and ischaemic conditions.

KEYWORDS

coagulation factor, endothelial tissue factor, haemorrhagic shock, paraoxonase-2, thrombin regulatory protein

1 | INTRODUCTION

Paraoxonase-2 (PON-2) is an enzyme with a molecular weight of 43 kDa and is widely expressed in cells. Subcellular distribution studies indicate that PON-2 is mainly located in the mitochondria and endoplasmic reticulum. PON-2-mRNA or protein has been detected in several tissues including the liver, lung, kidney, heart, pancreas, small intestine, muscle, and testis, and in endothelial cells, tracheal epithelial cells, and macrophages.[1] PON-2 has the highest hydrolytic activity of PON to many acyl-homoserine lactones, which mediate bacterial quorum sensing signals, and are important in regulating tissue factor (TF) expression and inducing host inflammatory responses.[2] In human endothelial cells, it has been shown that PON-2 indirectly but specifically reduces the release of superoxide in the mitochondrial inner membrane without affecting the levels of other free radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite.[3] Haemorrhagic shock leads to hypoperfusion of peripheral tissues and promotes endothelial dysfunction, which may lead to further tissue damage. Haemorrhagic shock can lead to post-traumatic coagulopathy.[4] Activation of coagulation plays an important role in thrombosis, which causes disseminated intravascular coagulation (DIC) associated with acute coronary artery disease and sepsis.[5] Coagulation occurs when blood is exposed to cells expressing endothelial TF.[6] TF is a physiological component of the subendothelial layer of blood vessels and surrounding tissues, forming a protective haemostatic lining that limits bleeding after vascular injury.[7] Unlike resting endothelial cells, activated endothelial cells can also express TF. TF binds to and activates factor VII, resulting in activation of factor X and eventual formation of thrombin.[8] We designed this study in order to investigate the molecular mechanism of PON-2 through endothelial TF to regulate coagulation activation in haemorrhagic shock rats.

Key Messages

- The molecular mechanism of paraoxonase-2 (PON-2) in regulating blood coagulation activation in rats with haemorrhagic shock through endothelial tissue factor was investigated.
- The expression of thrombomodulin in the haemorrhagic shock PON-2 group decreased significantly.
- Compared with group A, the activity of PON-2 in group B and group C was increased.
- PON-2 regulates the activation mechanism of coagulation in rats with haemorrhagic shock by regulating the expression of endothelial tissue-related genes.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and grouping

Thirty healthy male or female adult Sprague Dawley (SD) rats, weighing between 250 and 300 g each, from Weitong Lihua Co. (Beijing, China) were randomly divided into three groups: the control group (group A), the haemorrhagic shock PON-2 treatment group (group B), and the haemorrhagic shock group (group C), with ten rats in each group.

2.2 | Establishment of a haemorrhagic shock model

All rats were fasted one night before the experiment and were given free access to water. The rats were weighed before the experiment, and 5% chloral hydrate was intraperitoneally injected into rats at 6 mg/kg body weight. After rats became unconscious, the left common carotid artery and the right external jugular vein were separated. A 22-18G needle was used to pierce the artery and the arterial cannula was inserted. The entire system was prefilled with normal saline. The three-way and pressure sensors were connected to the multi-function monitor to detect real-time mean arterial pressure. Puncture and intubation were performed on the right external vein, which was mainly used for infusion and return of blood. No operation was performed in group A, and blood samples were taken from the inferior vena after 3 hours of observation. In groups B and C, after successful

intubation, rats were quickly bled until the mean arterial pressure dropped to 40 mmHg (1 mmHg-0.133 kPa). The mean arterial pressure was maintained at 40 mmHg for 60 minutes, which was a haemorrhagic shock model. The arteries were taken to save immediately.

2.3 | Preparation of serum

After the model was established, 1.8 mL of blood was withdrawn via the inferior vena cava of rats in each group using a disposable sterile syringe, and added to an anticoagulant plastic test tube containing 0.2 mL of sodium citrate, mixed and centrifuged at 3000 rpm. After 10 minutes, the plasma and serum were separated, and the supernatant was taken and stored at −70°C until use.

2.4 | Test indicators

Plasma thrombomodulin (TM), transcription factors (Egr-1) and (Sp1)

Plasma thrombin regulatory protein was determined by Western blotting assays. Plasma thrombin regulatory protein samples were negatively charged after sodium salt treatment, based on protein in polyacrylamide gels. The molecules moved from the cathode to the anode. Then, the film was transferred, and the separated band in the gel was transferred to the nitrocellulose membrane, and low voltage (100 V) and large current (1-2A) were selected, and the transfer was completed by energisation for 45 minutes. This was followed by enzyme immunolocalisation. The nitrocellulose membrane with the plasma TM band was sequentially bound to specific antibodies, and then horseradish peroxidase-conjugated TM, Egr-1, and Sp1 rat IgG were allowed to react with the primary antibody. The enzymatic reaction substrate-benzidine (brown), which formed an insoluble colour former, stained the zone. The positive reaction bands were clearly distinguishable. The membrane was washed with tris bufferedsaline-Tween and reacted with electrochemiluminescence reagent to expose the film, and the expression of plasma TM, Egr-1, and Sp1 was analysed.

Determination of TM-mRNA and PON-2-mRNA

The mRNA expression of TM and PON-2 in rat cells was determined by reverse transcription-polymerase chain Reaction (RT-PCR). Primer design was done using the DNASTAR software and was synthesised by Shanghai Biotech. Total RNA was extracted according to the instructions of the Trizol kit. Reverse transcription and PCR were performed according to Takara's instructions. The PCR system consisted of 3 μL of 25 mM magnesium sulfate, 8 μL of buffer, 0.5 μL of Taq, 27.5 μL sterile water, 4 μL of cDNA, 0.5 μL of target gene primer mixture, and 0.5 μL of β-actin primer mixture. PCR was carried out at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute for a total of 30 cycles, with a final extension for 5 minutes at 72°C. The amplified product was subjected to agarose gel electrophoresis. A gel imager was used to observe, analyse, and photograph mRNA expression. The primer sequences are shown in Table 1.

Detection of endothelial TF

TF was determined by enzyme linked immunosorbent assay (ELISA). The operation of this method was carried out in strict accordance with the kit instructions[1]: Set blank, standard and sample wells, add 100 μL to the bottom of the plate, and add 37°C, Incubate for 2 hours.[2] Discard the liquid and dry it, add 100 μL of biotin-labelled antibody working solution to each well, and incubate at 37°C for 1 hour.[3] Wash repeatedly three times with buffer, soak for 2 minutes each time, and dry.[4] One hundred microliters of horseradish peroxidase-labelled working fluid was added to each well and incubated at 37°C for 1 hour.[5] It was then washed repeatedly three times with buffer, soaked for 2 minutes each time, and dried.[6] Then, add the colour developer TMB and incubate at 37°C for 30 minutes in the dark.[7] Fifty microliters of the stop solution was sequentially added to the reaction well to terminate the reaction. The optical density value of each well was measured at 450 nm using a microplate reader.

Determination of PON-2 activity

The activity of PON-2 was determined by the phenyl acetate method. The reaction buffer was 50 mmol/L Tris pH 8.0 containing 2 mmol/L calcium chloride, and the ratio of phenyl acetate to absolute ethanol was 1:10. After mixing, appropriately mixed the diluted solution with the reflection buffer, added 10 μL of serum, started reaction with 1 mL of the substrate reaction solution, and immediately measured the change of the absorbance value of the reaction system within 5 minutes by colorimetry at 270 nm. The enzyme activity was calculated using the formula:

$$\text{PON-2 enzyme activity} = \frac{A \times 103 \times f \times Fv}{t \times \text{sv} \times L \times \varepsilon}$$

where A is the absorbance value, f is the dilution factor, Fv is the reaction final volume, t is the reaction time, sv

TABLE 1 Gene synthesis primer sequences

	Primer sequence	Amplification product length (bp)	Annealing temperature (°C)
TM	5-TCATCCTGGACGAGGGTTC-3	217	58
	5-GTCCGATTGCTTGATGGGT-3		
β-actin	5-AAAGAAAGGGTAAACGCA-3	488	58
	5-TCACGTCACTATCCCCAAT-3		

Abbreviation: TM, thrombomodulin.

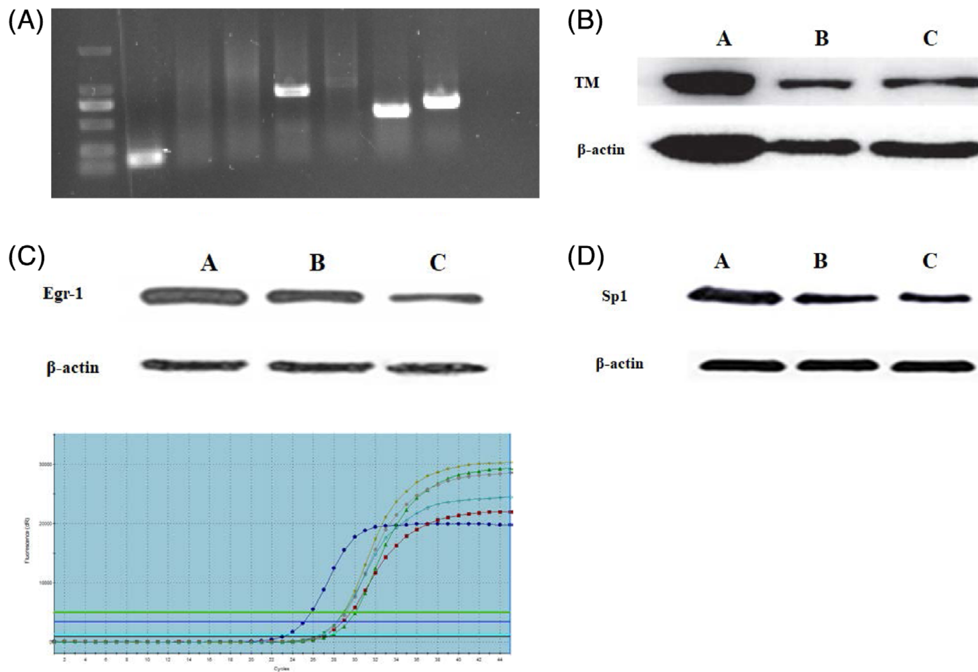

FIGURE 1 A, Serum gene expression in three groups of rats. B, Levels of TM expression. C, Levels of Egr-1 expression. D, Level of Sp1 expression. E, The amplification curve

is the sample volume, L is the light path, and ε is the molar extinction coefficient.

2.5 | Histopathological examination

The rat arteries were fixed in 4% paraformaldehyde to prepare paraffin sections of about 3 μm. Three randomly selected groups were used for haematoxylin and eosin staining. The changes of arteries in each group were observed under the light microscope at ×100.

2.6 | Statistical methods

The data in this study were processed using the SPSS20.0 statistical analysis software. Data were analysed by analysis of variance (ANOVA). The data were expressed as "mean ± SD." The statistical methods used included t test. $P < .05$ indicated statistically significant difference.

3 | RESULTS

3.1 | Comparison of the basic situation of the three groups of rats

In this study, 30 adult SD rats were selected, including 12 female rats and 18 male rats, with a mean body weight of 269.6 ± 11.2 g. They were randomly divided into three groups, which were healthy controls (Group A), the haemorrhagic shock PON-2 treatment group (group B), and the haemorrhagic shock group (group C). There were three female rats and seven male rats in group A with a mean body weight of 271.4 ± 8.9 g. There were four female rats and six male rats in group B, with a mean body weight of 268.1 ± 12.5 g. There were five female rats

TABLE 2 Serum TM protein expression levels in three groups of rats

Group	TM	Egr-1	Sp1
Healthy control group (group A)	279.33 ± 11.51	277.62 ± 11.35	281.62 ± 12.37
Haemorrhagic shock-injection of PON-2group (group B)	188.64 ± 12.47	185.23 ± 10.16	192.09 ± 11.45
Haemorrhagic shock group (group C)	137.48 ± 9.72[a,b]	125.83 ± 6.27[a,b]	119.49 ± 5.48[a,b]

Abbreviations: PON-2, paraoxonase-2; TM, thrombomodulin.
[a]Significant difference compared with the healthy control group, $P < .05$.
[b]Significant difference compared with the haemorrhagic shock-injection PON-2 group, $P < .05$.

TABLE 3 Expression levels of TM-mRNA and PON-2-mRNA in three groups of rats

Group	TM-mRNA	PON-2-mRNA
Healthy control group (group A)	0.78 ± 0.11	0.69 ± 0.34
Haemorrhagic shock-injection of PON-2group (group B)	0.97 ± 0.07[a]	0.86 ± 0.38[a]
Haemorrhagic shock group (group C)	1.14 ± 0.09[a,b]	1.12 ± 0.41[a,b]
F value	18.675	14.583
P value	.018	.013

Abbreviations: PON-2, paraoxonase-2; TM, thrombomodulin.
[a]Significant difference compared with the healthy control group, $P < .05$.
[b]Significant difference compared with the haemorrhagic shock-injection PON-2 group, $P < .05$.

and five male rats in group C with a mean body weight of 270.2 ± 5.8 g. There were no significant differences among the three groups ($P > .05$).

3.2 | Plasma TM detection, transcription factor (Egr-1), (Sp1)

Western blotting method was used to determine the content of TM. Compared with the healthy control group, the expression of TM in group B decreased significantly, and the expression of TM in group C also decreased significantly. There was significant difference between group B and C. The results are shown in Figure 1 and Table 2.

3.3 | Determination of TM-mRNA and PON-2-mRNA

The mRNA expression of TM and PON-2 in the serum of three groups of rats was detected by RT-PCR. The mechanism of PON-2 action could be directly reflected. The

TABLE 4 Expression levels of endothelial tissue factor in three groups of rats

Group	TF
Healthy control group (group A)	13.48 ± 1.23
Haemorrhagic shock-injection of PON-2 group (group B)	12.69 ± 1.07[a]
Haemorrhagic shock group (group C)	11.59 ± 0.87[a,b]
F value	12.378
P value	0.036

Abbreviations: PON-2, paraoxonase-2; TF, tissue factor.
[a]Significant difference compared with the healthy control group, $P < .05$.
[b]Significant difference compared with the haemorrhagic shock-injection PON-2 group, $P < .05$.

TABLE 5 Activity of paraoxonase-2 in three groups of rats

Group	PON-2
Healthy control group (group A)	28.38 ± 5.23
Haemorrhagic shock-injection of PON-2 group (group B)	110.34 ± 14.37[a]
Haemorrhagic shock group (group C)	52.37 ± 8.06[a,b]
F value	16.415
P value	0.007

Abbreviation: PON-2, paraoxonase-2.
[a]Significant difference compared with the healthy control group, $P < .05$.
[b]Significant difference compared with the haemorrhagic shock-injection PON-2 group, $P < .05$.

mRNA TM expression was 0.78 ± 0.11 in group A and 0.97 ± 0.07 in group B. There was significant difference between group A and B. The expression of mRNA TM was 1.14 ± 0.09 in group C, which was significantly higher than in groups A and B. There were significant differences among the three groups (Table 3).

The mRNA expression of PON-2 in the three groups was compared. The mRNA expression of PON-2 was 0.69 ± 0.34 in group A, and 0.86 ± 0.38 in group B. Compared with group A, there was a significant difference. The mRNA expression level of PON-2 in group C

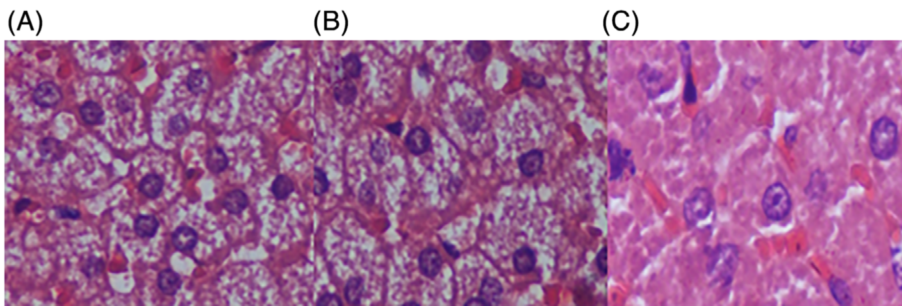

FIGURE 2 Comparison of arterial sections of three groups of rats

was 1.12 ± 0.41, which was significantly different from groups A and B, and there were significant differences among the three groups.

3.4 | Determination of endothelial TF

The expression of endothelial TF in serum of the three groups was determined by ELISA. Compared with group A, the expression of endothelial TF decreased in groups B and C, and there was a significant difference. The expression of endothelial TF was different between groups B and C. The results are shown in Table 4.

3.5 | Determination of PON-2 activity

The activity of PON-2 in the serum of the three groups of rats was measured. The results showed that compared with group A, the activity of PON-2 in groups B and C was increased, and there were significant differences. Compared with groups B and C, the activity of PON-2 was also different. The results are shown in Table 5.

3.6 | Histological examination

By observing the sections of the arterial endothelium, it was found that the nuclei of groups A and B were clearly visible, in blue, with large nuclei and intact edges, while group C had haemorrhage and apoptosis. The result is shown in Figure 2.

4 | DISCUSSION

Haemorrhagic shock is a syndrome in which blood is circulated and blood supply is insufficient, tissue hypoxia and hypoperfusion, followed by DIC and multiple organ dysfunction syndrome.[9] In the process of haemorrhagic shock, due to hypoxia, ischaemia, and the release of oxygen free radicals, endothelial cells, which have the functions of regulating blood coagulation and anticoagulation, are damaged.[10] Under normal conditions, vascular endothelial cells release antithrombotic substances to prevent formation of blood clots,[11] and when the blood vessels are damaged, vascular endothelial cells secrete tissue-like factors such as tissue-stimulating substances, thereby acting as coagulation.[12] Plasma thrombin is a type of transmembrane glycoprotein on the surface of vascular endothelial cells. TM binds to thrombin to activate protein C, which acts as an anticoagulant.[13]

In this experiment, the expression of TM and transcription factors Egr-1 and Sp1 was determined by Western blotting assays. Compared with healthy controls, haemorrhagic shock caused a decrease in the expression levels of TM, Egr-1, and Sp1. After the blood vessel is damaged, protein C needs to be converted into activated protein C, which consumes more TM, or affects the synthesis of TM.[14] This results in a decrease in the expression of TM. The mRNA expression of TM was measured, and the mRNA expression of TM in groups B and C was elevated. The main reason was that after the blood vessel was damaged, a large amount of TF entered the blood, and platelets were activated, resulting in decreased number of blood cells, and formation of a large amount of thrombin, which increased the mRNA expression of TM.[15] The changes of endothelial TF in serum determined by ELISA showed that haemorrhagic shock increased the expression of TF. There were significant differences in the expression of TF among the three groups. It indicated that endothelial cells secreted more TF and played a role in coagulation after vascular injury caused by hypoxia and ischaemia.[16] By measuring the expression of PON-2 and the enzyme activity, it was found that the PON-2-mRNA and enzyme activities in groups B and C were increased compared with the healthy control group and there were significant differences. It was indicated that when the body was under hypoxic–ischaemic conditions, PON-2 protected blood vessels through its antioxidant capacity and inhibited the release of endothelial TF from blood vessels, thereby acting as a blood coagulant.[17] The mitochondria are a major

source of free radical-associated oxidative stress, and the dominant localisation of PON-2 in the mitochondria will support the role of this enzyme in protecting cells from oxidative damage.[18] PON2 has been shown to be indirectly but specifically reduced in human endothelial cells from superoxide from the mitochondrial inner membrane, without affecting the levels of other free radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite.[19,20] Endothelial cell TF expression further enhanced and maintained blood vessels including procoagulants and proinflammatory signalling. Pathological inflammation-coagulation pathway, mainly driven by thrombosis, can alter RNA or TF splicing through post-transcriptional regulation of TF.

PON-2 regulates the activation mechanism of coagulation in rats with haemorrhagic shock by regulating the expression of endothelial tissue-related genes such as plasma TM transcription factors (Egr-1) and (Sp1), and endothelial TF under hypoxic and ischaemic conditions.

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