Upregulation of Proteinase-Activated Receptor-2 and Increased Response to Trypsin in Endothelial Cells after Exposure to Oxidative Stress in Rat Aortas

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Key Words

Endothelial cell • Proteinase-activated receptor • Rat aorta • Vasodilation • Oxidative stress • Trypsin

Abstract

Background/Aims: The effects of oxidative stress on the vascular responsiveness to the agonists of proteinase-activated receptors (PARs) were investigated. **Methods:** Serum-free incubation was utilized to impose oxidative stress to isolated rat aortas. Spontaneously hypertensive rats (SHR) were investigated as a model of in vivo oxidative stress. **Results:** Thrombin, trypsin, PAR₁-activating peptide (PAR₁-AP), PAR₂-AP and PAR₄-AP induced little or no effect in the aortas of female Wistar-Kyoto rats (WKY). Serum-free incubation induced endothelium-dependent relaxant responses to PAR₂ agonists, but not PAR₁ or PAR₄ agonists, in a manner sensitive to diphenyleneiodonium or ascorbic acid. In male aortas, trypsin and PAR₂-AP induced a transient endothelium-dependent relaxation without serum-free incubation. The acetylcholine-induced endothelium-dependent relaxation and the sodium nitroprusside-induced endothelium-independent relaxation remained unchanged. Immunoblot analyses revealed the upregulation of PAR₂ in endothelial cells, which was abolished by either diphenyleneiodonium or ascorbic acid. Aortas of female SHR expressed a higher level of PAR₂

than WKY and responded to trypsin without serum-free incubation. Treatment with ascorbic acid attenuated the trypsin-induced relaxation and the PAR₂ expression in SHR. **Conclusion:** This study provides the first evidence that oxidative stress upregulates PAR₂ in endothelial cells, thereby enhancing the endothelium-dependent relaxant response to PAR₂ agonists in rat aortas.

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Introduction

Proteinase-activated receptors (PARs) form a unique family of G protein-coupled receptors which mediate the cellular effects of serine proteinases such as thrombin and trypsin [1–3]. The activation of PARs is initiated by proteolytic cleavage of the extracellular region at the specific site [1, 2]. Therefore, one proteinase could activate multiple receptors, while one type of receptor could be activated by multiple proteinases. Among the 4 members of PARs, PAR₁, PAR₃ and PAR₄ serve as receptors for thrombin, while PAR₁, PAR₂ and PAR₄ serve as receptors for trypsin [1–3]. In terms of thrombin or trypsin receptor, PAR₂ serves as a specific receptor for trypsin. However, the role of PAR₁ as a trypsin receptor is controversial [2, 4, 5]. Trypsin has been shown to cleave and inactivate

PAR₁ in rat myometrium and vascular endothelial cells [4, 5], while it was also reported to activate PAR₁ at relatively higher concentrations than those required to activate PAR₂ [2].

The different sets of PARs are expressed in a tissue-specific manner and mediate the tissue-specific responses to agonist proteinases [1, 2]. In vascular tissues, endothelium-dependent relaxation is the most frequently reported vascular effect of thrombin and trypsin, while endothelium-dependent contraction or direct smooth muscle contraction has also been reported depending on the type of blood vessels [3, 6–8]. In addition to the vasomotor function, PAR₁ stimulation has been reported to induce endothelial barrier dysfunction, production of reactive oxygen species, cytokine release and alteration of the expression of various genes, including cell adhesion molecules and tissue factor [1–3]. PAR₂ stimulation has also been reported to induce cytokine release, leukocyte adhesion and exocytosis of a Weibel-Palade body in endothelial cells [9–12]. These effects mediated by PAR₁ and PAR₂ are thus suggested to contribute to the pathogenesis and pathophysiology of vascular diseases.

Various chemical factors and physical stimulations as well as pathological conditions have been shown to modulate the expression of PARs [3, 13]. For example, the expression of PAR₁ has been reported to be upregulated by the action of growth factors or in vascular lesions of balloon injury, atherosclerosis and subarachnoid hemorrhage [14–20]. On the other hand, the PAR₂ expression has been reported to be upregulated by inflammatory stimuli such as tumor necrosis factor- α , interleukin-1 α and 1 β and lipopolysaccharide [21–23]. The upregulation of PARs is considered to be a critical step for these receptors to contribute to the pathogenesis and pathophysiology of vascular diseases, especially for those expressed at a low level under physiological conditions [3]. On the other hand, oxidative stress is one of the critical factors contributing to the pathogenesis and pathophysiology of vascular disease, such as hypertension, vasospasm, atherosclerosis and subarachnoid hemorrhage [24–26]. It is therefore suggested that the modulation of the expression of PARs is one of the mechanisms underlying the pathological effects of oxidative stress in vascular diseases. Cyclic strain has been reported to upregulate PAR₁ in smooth muscle in a manner sensitive to a NADPH oxidase inhibitor [27]. However, the effects of oxidative stress on the responses to PARs agonists and the expression of PARs in vascular tissues still remain to be investigated.

The present study thus investigated the effect of oxidative stress on the vascular responsiveness to thrombin,

trypsin, PAR₁-activating peptide (PAR₁-AP) and PAR₂-AP in vascular tissues. For this purpose, 24-hour incubation in serum-free media was utilized to impose oxidative stress to the isolated vascular tissues, as previously reported [28, 29]. Spontaneously hypertensive rats (SHR) have been reported to be exposed to a higher degree of oxidative stress than Wistar-Kyoto rats (WKY) [30]. SHR were thus investigated as a model of in vivo oxidative stress. Since the responsiveness to the stimulation of PAR₂, but not PAR₁ or PAR₄, was observed after serum-free incubation, the expression of PAR₂ was then directly investigated by an immunoblot analysis. The present study thereby demonstrates, for the first time, that oxidative stress upregulates the PAR₂ expression and enhances the response to the PAR₂ activation in vascular endothelial cells.

Methods

Tissue Preparation

WKY (Kyudo, Saga, Japan) and SHR (Charles River, Yokohama, Japan), ranging in age from 9 to 11 weeks, were euthanized by intraperitoneal injection of 100 mg sodium pentobarbital/kg weight, according to the protocol approved by the Animal Care and the Ethical Committee of Kyushu University. The study was performed mainly with virgin female rats, unless otherwise specified. The descending thoracic aorta was excised and the adventitial tissues were mechanically removed under a binocular microscope. The aortas were opened longitudinally and then cut into circular strips measuring ~ 1 mm in width and ~ 4 mm in length. The strips with an intact endothelium were mainly used in the study, unless otherwise specified. When the study was conducted in the absence of endothelium, the luminal surface was rubbed off with a cotton swab to remove the endothelium. The vascular strips were equilibrated in a physiological saline solution (PSS) consisting of 123 mmol/l NaCl, 4.7 mmol/l KCl, 1.25 mmol/l CaCl₂, 1.2 mmol/l MgCl₂, 1.2 mmol/l KH₂PO₄, 15.5 mmol/l NaHCO₃ and 11.5 mmol/l D-glucose, aerated with 95% O₂ and 5% CO₂.

Protocol for Imposing Oxidative Stress to the Isolated Strips

The strips were incubated for 24 h at 37°C in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics in a CO₂ incubator, as previously reported [28, 29]. The strips were then thoroughly washed and equilibrated in PSS before starting the experimental protocols. For the control, the strips were used in the experiments on the day of preparation and without incubation in serum-free DMEM at 37°C for 24 h. These control strips were kept in PSS at room temperature until use.

Tension Measurement of the Isolated Strips

The strips were mounted vertically in an organ bath and connected to a force transducer (TB612-T, Nihon Koden, Japan), as previously described [31]. The change in the tension was then monitored at 37°C under a 300-mg resting load. The endotheli-

um-dependent relaxant responses were examined during the sustained phase of the pre-contraction induced by 100 nmol/l U46619, a thromboxane A₂ analogue. The value of tension was expressed as a percentage, assigning the levels of tension at rest and during the contraction induced by 100 nmol/l U46619 to be 0 and 100%, respectively.

Acetylcholine was used as a control stimulation to induce an endothelium-dependent relaxation in rat aortas. In the strips of female WKY aortas before serum-free incubation, 10 μ mol/l acetylcholine induced an $\sim$ 20% reduction in the U46619-induced pre-contraction. This relaxant effect was weaker than the previously reported relaxant effect of acetylcholine in female rat aortas ($\sim$ 70–90% reduction) [32–34]. However, preliminary experiments revealed that this weak response to acetylcholine was not due to usage of vascular strips, because 10 μ mol/l acetylcholine induced a similar relaxation during the U46619-induced pre-contraction in ring preparations ($28.1 \pm 7.7\%$ reduction of the pre-contraction in ring preparations vs. $26.9 \pm 4.9\%$ reduction in vascular strips, $n = 4$). On the other hand, during pre-contraction induced by 10 μ mol/l phenylephrine, 10 μ mol/l acetylcholine induced a $58.9 \pm 9.6\%$ reduction in tension in ring preparations and $46.1 \pm 1.6\%$ reduction in vascular strips ($n = 4$). Acetylcholine was reported to induce 50–90% relaxation during the phenylephrine-induced pre-contraction, depending on the report [32–38]. Therefore, the relaxant effect seen during the phenylephrine-induced contraction was consistent with the lower side of the reported values [35–38].

Immunoblot Analysis of the Expression of PAR₂ and eNOS

The tissue lysates were prepared in the lysis buffer consisting of 50 mmol/l Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mmol/l NaCl, 10 mmol/l MgCl₂, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ mol/l 4-aminidophenylmethane sulfonyl fluoride, as previously reported [15]. Total protein (20 μ g) was subjected to an immunoblot analysis with a mouse monoclonal anti-PAR₂ antibody (sc-13504, SAM11; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and a polyclonal anti-PAR₂ antibody (sc-5597, H99; Santa Cruz Biotechnology) at 0.2 and 0.4 μ g/ml, respectively, and a horseradish peroxidase-conjugated secondary antibody (Sigma, St. Louis, Mo., USA) in an immunoreaction enhancer solution (Can-Get-Signal; Toyobo, Osaka, Japan). The expression of endothelial NO synthase (eNOS) was detected with anti-eNOS antibody (No. 610296; BD Biosciences, San Jose, Calif., USA) at 250-fold dilution. The immune complex was detected with an enhanced chemiluminescence technique (GE Healthcare, Tokyo, Japan). The chemiluminescence signal was detected and analyzed with the ChemiDoc XRS-J image analysis system (Bio-Rad, Tokyo, Japan). After performing immunoblot detection, the membranes were stained with naphthol blue black to visualize the actin bands. As shown in figure 8c, the level of actin did not significantly differ between the presence and absence of endothelium, and also before and after serum-free incubation, thus suggesting a negligible contribution of the endothelial actin to the level of total actin. The levels of eNOS and PAR₂ were therefore evaluated by normalizing the sample loading by the level of actin.

N-Glycosidase F Treatment

The tissue lysates were treated with and without (for control) 7.5 units N-glycosidase F/10 μ g protein at 37°C for 3 h, according

to the manufacturer's instruction. Thereafter, the lysates were subjected to an immunoblot analysis for PAR₂ as described above.

Drugs and Solutions

Thrombin (bovine plasma), trypsin (bovine pancreas), DMEM, acetylcholine, sodium nitroprusside (SNP), diphenyleneiodonium chloride (DPI), N^ω-nitro-L-arginine methyl ester (L-NAME), U46619 and N-glycosidase F were purchased from Sigma. Superoxide dismutase was from Calbiochem (La Jolla, Calif., USA). TFLLR-NH₂ (PAR₁-AP), SLIGRL-NH₂ (PAR₂-AP), and AYPGKF-NH₂ (PAR₄-AP) were from Bachem (Bubendorf, Switzerland). L(+)-ascorbic acid was from Wako (Osaka, Japan).

Statistical Analysis

All data are expressed as the mean $\pm$ SEM of the indicated number of experiments. One strip obtained from one animal was used for each experiment, and therefore the number of experiments indicates the number of animals. Unpaired Student's t-test and a one-way ANOVA followed by Scheffé's post hoc test were used to determine any significant differences. A value of $p < 0.05$ was considered to be statistically significant.

Results

Enhancement of the PAR₂-Mediated Relaxation after 24-Hour Incubation in Serum-Free Media in WKY Aortas

In the strips with endothelium obtained from the female WKY aortas and before incubating in serum-free media, 100 nmol/l U46619 induced a sustained contraction (fig. 1a, d). Trypsin and PAR₂-AP had no effect on this contraction (fig. 1a, d). After 24-hour incubation in serum-free media at 37°C, trypsin and PAR₂-AP induced a significant relaxation in rat aortas (fig. 1b, e, 2e). The sustained phase of relaxation was frequently accompanied by oscillatory responses (fig. 1b, e). When incubated at 4°C in either serum-free DMEM or PSS for 24 h, trypsin and PAR₂-AP induced no responses (fig. 1c, f). On the other hand, after 24-hour incubation with 5% serum, trypsin and PAR₂-AP induced 28.5 and 25.6% reductions ($n = 2$) in the U46619-induced pre-contraction, respectively. It is therefore conceivable that ex vivo incubation at 37°C but not serum deprivation was responsible for the observed changes in the PAR₂-mediated responses. The changes in the relaxant responses were therefore compared between before and after serum-free incubation.

The relaxations seen with trypsin and PAR₂-AP after serum-free incubation were abolished either by the treatment with L-NAME or the removal of endothelium (fig. 2). L-NAME was applied 30 min before initiating the pre-contraction by U46619. The endothelium was removed after 24-hour serum-free incubation of the strips

Fig. 1. Induction of the relaxant response to PAR₂ agonists after 24-hour incubation in serum-free media in female WKY aortas. Representative recordings of the response to trypsin (**a–c**) or PAR₂-AP (**d–f**) during the U46619-induced contraction in the strips with an endothelium, before (**a**, **d**) and after 24-hour incubation in serum-free media at 37° (**b**, **e**) and 4°C (**c**, **f**). The levels of tension obtained at rest and just prior to application of trypsin or PAR₂-AP during the U46619-induced contraction were assigned values of 0 and 100%, respectively. Similar results were observed in 5 (**a**, **b**, **d**, **e**) and 3 (**c**, **f**) independent experiments.

with endothelium. The successful removal of functional endothelium was confirmed by the loss of the relaxant response to acetylcholine (fig. 2b, d). In female WKY aortas, acetylcholine induced a relaxant response in the presence of endothelium (fig. 5). The evaluation of the concentration-dependent responses revealed that the relaxant response to trypsin was significantly enhanced after the incubation in serum-free media (fig. 3). In contrast, the relaxant response to SNP, a NO donor, remained unaltered (fig. 3).

In male WKY aortas, trypsin and PAR₂-AP induced a transient but significant relaxation without serum-free incubation (fig. 4). However, this relaxation was similarly enhanced by 24-hour serum-free incubation as observed

with female aortas and became a sustained relaxation (data not shown). Since female rat aortas did not respond to PAR₂ agonists without serum-free incubation, while the enhancement of the relaxant response to PAR₂ agonists was similarly observed in males and females, female rats were primarily used in the present investigation.

Acetylcholine induced an endothelium-dependent relaxation in female WKY aortas, which remained unchanged after serum-free incubation (fig. 5a, b). Thrombin, even at 3 units/ml, had no effect on the U46619-induced contraction both before and after 24-h incubation in serum-free media (fig. 5c, d). This concentration of thrombin has been shown to be sufficient for inducing endothelium-dependent responses in other arteries

Fig. 2. Requirement of endothelium and nitric oxide for the PAR₂-mediated relaxant response seen after 24-hour incubation in serum-free media in female WKY aortas. Representative recordings of the response to trypsin (**a, b**) or PAR₂-AP (**c, d**) during the U46619-induced contraction, either in the presence of endothelium and 100 μ mol/l L-NAME (Endothelium (+) + L-NAME; **a, c**) or in the absence of endothelium (Endothelium (-); **b, d**), after 24-hour incubation in serum-free media at 37°C. For the experiments in the absence of endothelium, strips with an intact endothelium were incubated in serum-free media, and then the endothelium was removed. Stimulation with acetylcholine (ACh) was used to confirm a loss of functional endothelium. **e** Summary of the responses to trypsin and PAR₂-AP, before and after serum-free incubation, and also in the presence of L-NAME (After, E(+) + L-NAME) or in the absence of endothelium (After, E(-)). The levels of tension obtained at rest and just prior to application of trypsin or PAR₂-AP during the U46619-induced contraction were assigned values of 0 and 100%, respectively. The data are presented as the mean $\pm$ SEM of the indicated number of experiments. * $p < 0.05$.

[8, 39]. PAR₁-AP (100 μ mol/l) induced an additional small contraction before incubating in serum-free media, both with (fig. 5e) and without an endothelium (data not shown), thus indicating that the contractile effect of PAR₁-AP was a direct effect on smooth muscle. A similar response to PAR₁-AP was also observed after serum-free incubation (fig. 5f). PAR₄-AP, up to 200 μ mol/l, had no apparent effect under any conditions (fig. 5g, h).

DPI and Ascorbic Acid Prevented the Enhancement of the PAR₂-Mediated Relaxation after 24-Hour Incubation in Serum-Free Media in WKY Aortas

The involvement of oxidative stress in the enhancement of the PAR₂-mediated relaxant response seen after

serum-free incubation was evaluated by using DPI as an inhibitor of NADPH oxidase and ascorbic acid as an antioxidative agent. The inclusion of 1 μ mol/l DPI or 1 mmol/l ascorbic acid during the 24-hour incubation in serum-free media substantially and significantly suppressed the induction of relaxant responses to trypsin and PAR₂-AP (fig. 6). However, DPI and ascorbic acid had no significant effect on the acetylcholine-induced relaxation (fig. 6), thus ruling out the nonspecific inhibitory effect or a direct inhibition of eNOS activity by DPI and ascorbic acid. Superoxide dismutase (500 units/ml) exhibited no preventive effects on the induction of the relaxant response to trypsin (fig. 6). However, 24-hour serum-free incubation with 10–1,000 units/ml catalase totally impaired the contractility of the artery (data not

Fig. 3. Concentration-dependent relaxant effects of trypsin and sodium nitroprusside (SNP) in female WKY aortas, before and after 24-hour serum-free incubation. The relaxant effects of trypsin and SNP were evaluated during the U46619-induced contraction in the strips with endothelium, before and after 24-hour incubation in serum-free media at 37°C. The response to SNP was evaluated in the presence of 100 $\mu\text{mol/l}$ L-NAME. The levels of tension obtained at rest and just prior to the application of trypsin or SNP during the U46619-induced contraction were assigned values of 0 and 100%, respectively. The data are presented as the mean $\pm$ SEM (n = 4–5). * p < 0.05 vs. before.

Fig. 4. Relaxant response to PAR₂ agonists in male WKY aortas without serum-free incubation. Representative recordings and a summary of the relaxant effect of trypsin and PAR₂-AP during the U46619-induced contraction in the strips with endothelium of male WKY aorta obtained without 24-hour incubation in serum-free media. The data for female aortas were obtained from

the experiments shown in figure 1. The levels of tension obtained at rest and just prior to application of trypsin or PAR₂-AP during the U46619-induced contraction were assigned values of 0 and 100%, respectively. The data are presented as the mean $\pm$ SEM (n = 3 for male; n = 5 for female). * p < 0.05 vs. 100% level.

shown). Therefore, the effect of catalase on the induction of the relaxant response to PAR₂ agonists was not examined.

Relaxant Response to Trypsin in SHR

To establish the physiological relevance of the in vitro observations, the relaxant response to trypsin was examined in SHR, which has been reported to be exposed to a higher level of oxidative stress in vivo than WKY [30]. We thus hypothesized that trypsin would induce an endothelium-dependent relaxation in the SHR aortas without serum-free incubation. As shown in figure 7, trypsin did

induce a transient relaxation in the SHR aortas without serum-free incubation. However, serum-free incubation further augmented the relaxant response to trypsin in SHR aortas as observed with WKY aortas and converted a transient response to a sustained response (data not shown). Notably, intraperitoneal treatment with 3 g ascorbic acid/kg weight/day for 3 days attenuated the relaxant response to trypsin (fig. 7).

Upregulation of the Expression of PAR₂ in Rat Aortas

Since the response to PAR₂ agonists was observed to be selectively augmented after exposure to oxidative

Fig. 5. Effect of 24-hour incubation in serum-free media on the response to acetylcholine (ACh), thrombin, PAR₁-AP and PAR₄-AP in female WKY aortas. Representative recordings showing the responses to ACh (**a, b**), thrombin (**c, d**), PAR₁-AP (**e, f**) and PAR₄-AP (**g, h**) during the U46619-induced contraction in the strips with endothelium, before (**a, c, e, g**) and after (**b, d, f, h**) 24-hour incubation in serum-free media at 37°C. The levels of tension obtained at rest and just prior to the application of ACh, thrombin, PAR₁-AP or PAR₄-AP during the U46619-induced contraction were assigned values of 0 and 100%, respectively. Similar results were observed in 3 independent experiments.

Fig. 6. Effects of diphenyleneiodonium, ascorbic acid and superoxide dismutase on the response to trypsin, PAR₂-AP and acetylcholine after 24-hour incubation in serum-free media in WKY aortas. Summary of the relaxant responses to trypsin, PAR₂-AP and acetylcholine (ACh) during the U46619-induced contraction in the strips with endothelium, after 24-hour incubation in serum-free media at 37°C without and with 1 μ mol/l diphenyleneiodonium (+DPI), 1 mmol/l ascorbic acid (+Ascorbic acid) or 500 units/ml superoxide dismutase (+SOD). The levels of tension obtained at rest and just prior to application of trypsin, PAR₂-AP or ACh during the U46619-induced contraction were assigned values of 0 and 100%, respectively. The data are presented as the mean $\pm$ SEM (n = 3–5). * p < 0.05 vs. control.

Fig. 7. The relaxant response to trypsin in female SHR aortas. Representative recording and a summary of the relaxant effect of trypsin during the U46619-induced contraction in the strips of the SHR aortas without 24-hour incubation in serum-free media. SHR were either untreated (SHR) or treated (SHR + As) with intraperitoneal injection of 3 g ascorbic acid/kg weight/day for 3 days. The data for female WKY aortas were obtained from the experiments shown in figure 1. The levels of tension obtained at rest and just prior to application of trypsin were assigned values of 0 and 100%, respectively. The data are presented as the mean $\pm$ SEM (n = 5 for SHR; n = 5 for WKY). * p < 0.05.

stress both in vitro and in vivo, the expression of PAR₂ was then investigated with an immunoblot analysis to correlate the increased responsiveness to any change in the receptor expression. A monoclonal anti-PAR₂ antibody SAM11 detected two major bands of 55 and 71 kDa (fig. 8a, d, e). These bands were also detected with a polyclonal anti-PAR₂ antibody H99 (fig. 8d), and this detection pattern was similar to that in the previous reports [40, 41]. However, antibody H99 required longer exposure to yield the image in chemiluminescence detection despite a higher concentration, and also resulted in weaker bands with a higher background (fig. 8d). We therefore used SAM11 in the present study to evaluate the expression of PAR₂. N-Glycosidase F treatment has been shown to work by removing the glycosylation of the mature PAR₂, thereby reducing the size of immunoreactive bands to 33–48 kDa [42]. Accordingly, we examined the effect

of N-glycosidase treatment on the pattern of immunodetection. However, this was not the case in the present study (fig. 8e). As a result, the levels of two bands detected by antibody SAM11 and their sum were evaluated as an indication of the level of PAR₂. They exhibited similar changes, despite some difference in the statistical significance (fig. 8f).

The PAR₂ expression was detected in the strips of WKY aortas with an endothelium before incubating in serum-free media (fig. 8f; Before, E(+)). This level was not affected by removing the endothelium (fig. 8f; Before, E(-)). After 24-hour serum-free incubation, the level of PAR₂ was significantly upregulated (fig. 8f; After, E(+)), while this upregulation was abolished by removing the endothelium (fig. 8f; After, E(-)). However, the level of eNOS remained unchanged after serum-free incubation (fig. 8b). The successful removal of the endothelium after

Fig. 8. Immunoblot analysis of the expression of PAR₂ and eNOS in the aortas of female WKY and SHR. Representative immunoblot (a) and summary of the analysis of the expression of eNOS (b), actin (c) and PAR₂ (f) in the aortas with (E(+)) and without (E(-)) endothelium. d The comparison of the detection patterns between two anti-PAR₂ antibodies; SAM11 (0.2 µg/ml) and H99 (0.4 µg/ml). e The effect of N-glycosidase F treatment on the detection pattern of anti-PAR₂ antibody SAM11. The samples of WKY were obtained either before or after 24-hour incubation in serum-free media at 37°C without (After) and with 1 µmol/l diphenyleneiodonium (After + DPI) or 1 mmol/l ascorbic acid (After + As). The samples of the SHR were obtained without serum-free incubation (SHR). The expression levels of 71- and 55-kDa proteins were evaluated either separately or as a sum (71 + 55 kDa). The data are presented as the mean ± SEM (b, c n = 3; f n = 3–5). * p < 0.05 vs. Before, E(+); † p < 0.05 vs. After, E(+).

Fig. 9. Immunoblot analysis of the expression of PAR₂ in the aortas of female SHR with and without intraperitoneal treatment with ascorbic acid. The level of PAR₂ expression evaluated as a sum of 71- and 55-kDa bands in the aortas of female SHR rats with (E(+)) and without (E(-)) endothelium, and with (SHR + As) and without (SHR) intraperitoneal injection of 3 g ascorbic acid/kg weight/day for 3 days. The levels of PAR₂ are expressed as a percentage of those seen with SHR, E(+). The data are presented as the mean $\pm$ SEM (n = 3). * p < 0.05; n.s. = not significantly different.

endothelial denudation was also validated by the near-complete loss of the eNOS expression (fig. 8a). The level of PAR₂ in the strips of SHR aortas without serum-free incubation (fig. 8f; SHR, E(+)) was higher than that seen in WKY aortas before serum-free incubation (fig. 8f; Before, E(+)) and similar to that seen in the WKY aortas after serum-free incubation (fig. 8f; After, E(+)). Treatment with 1 μ mol/l DPI or 1 mmol/l ascorbic acid completely prevented the upregulation of PAR₂ seen after serum-free incubation in the WKY aortas (fig. 8f; After + DPI, E(+) and After + As, E(+)).

In SHR, the endothelial denudation also significantly decreased the level of PAR₂ to a similar extent seen in the WKY aortas after serum-free incubation (fig. 9). The intraperitoneal treatment of SHR with 3 g ascorbic acid/kg weight/day for 3 days decreased the level of PAR₂ to that seen after endothelium denudation (fig. 9).

Discussion

The major findings of the present study are: (1) in WKY rat aortas, the endothelium-dependent relaxant responses to trypsin and PAR₂-AP were augmented by serum-free incubation in a manner sensitive to antioxidative agents, DPI and ascorbic acid; (2) in SHR aortas, trypsin induced an endothelium-dependent relaxation without serum-free incubation and this relaxant response was attenuated by intraperitoneal treatment with ascorbic acid; (3) the expression of PAR₂ in endothelial cells was upregulated in WKY rat aortas after serum-free incubation in a manner sensitive to DPI and ascorbic acid, while the expression of eNOS remained unchanged; (4) the level of PAR₂ in SHR was higher than that seen in WKY before serum-free incubation and this level of PAR₂ decreased after intraperitoneal treatment with ascorbic acid, and (5) the augmenting effect of serum-free incubation was specific to the PAR₂-mediated responses, while the responses to thrombin, PAR₁-AP, PAR₄-AP and acetylcholine remained unaffected. These in vitro and in vivo observations thus suggest that the relaxant responses to PAR₂ agonists were enhanced by oxidative stress due to the upregulation of the expression of PAR₂ in endothelial cells. The cyclic strain has been shown to upregulate the expression of PAR₁ in human aortic smooth muscle in a manner sensitive to a NADPH oxidase inhibitor [27]. The present study thus provides the first evidence that oxidative stress upregulates the expression of PAR₂ in endothelial cells.

The oxidative stress was suggested to upregulate PAR₂ in endothelial cells but not smooth muscle, because (1) the relaxation induced by trypsin and PAR₂-AP was abolished in the absence of endothelium; (2) no contractile response to trypsin or PAR₂-AP was observed in the WKY aortas after 24-hour serum-free incubation or in the SHR aortas, and (3) the removal of the endothelium abolished the upregulation of PAR₂, while it had no effect on the level of PAR₂ seen before incubating in serum-free media. The observations also indicated that PAR₂ was expressed in the smooth muscle of the WKY aortas before serum-free incubation, and this expression was not changed by oxidative stress. The reason why PAR₂ stimulation induced no apparent contraction remains unknown. The level of PAR₂ on the cell surface of smooth muscle may therefore be insufficient to induce any contraction.

The observation that the relaxant effect of SNP remained unchanged after exposure to oxidative stress suggests that the enhanced relaxant response to trypsin seen after serum-free incubation is attributable to an increased

production of NO, but not to an increased responsiveness of smooth muscle to the relaxant effect of NO. The relaxant response to acetylcholine remained unaffected after serum-free incubation, thus ruling out the general augmentation of NO production after the exposure to oxidative stress. The increased relaxant effect of trypsin is thus suggested to be attributable to the increased function of PAR₂, which is mainly due to the upregulation of the PAR₂ expression.

PAR₄-AP, <200 µmol/l, had no effect on the contraction either before or after serum-free incubation. This observation is apparently inconsistent with the observations in the previous report, which showed an endothelium-dependent relaxant effect of two PAR₄-APs in rat aortas [43]. However, PAR₄-APs used in the previous report, GYPGQV-NH₂ and GYPGKF-NH₂, were different from those used in the present study (AYPGKF-NH₂), and required concentrations of >100 µmol/l to induce the relaxant effect [43]. PAR₄-AP used in the present study has been shown to induce NO production in the cultured vascular endothelial cells at the concentrations of <100 µmol/l [44]. Our observations therefore suggest that PAR₄ plays a negligible role, if any, in the endothelium-dependent relaxation in the female rat aorta of WKY. However, the degree of acetylcholine-induced relaxation seen during the U46619-induced pre-contraction was relatively smaller than that in the literature [32–34]. Therefore, underestimation of the relaxant effect of PAR₄-AP could not be fully ruled out.

DPI is a flavoprotein inhibitor and therefore inhibits the activity of eNOS as well as NADPH oxidase, thereby inhibiting the NO production and also the acetylcholine-induced relaxation in rat aortas [45–47]. The observation that DPI inhibited the induction of the relaxant response to trypsin may suggest NO but not reactive oxygen species to be responsible for this induction. However, the treatment with ascorbic acid also prevented the induction of the relaxant response to trypsin after serum-free incubation. Ascorbic acid does not inhibit the NO production but instead increases the bioavailability of NO [48]. It is therefore unlikely that NO is responsible for the induction of the relaxant response to trypsin. On the other hand, eNOS could produce reactive oxygen species when eNOS is ‘uncoupled’ [24, 46]. DPI inhibits this production of reactive oxygen species [46]. Therefore, the observation with DPI suggests a possible role of eNOS as a source of oxidative stress during serum-free incubation.

However, precisely how such oxidative stress was generated during serum-free incubation still remains to be elucidated. Oxidative stress is generated in any cell type

as a consequence of normal cellular metabolism and lipid membrane turnover [29, 49]. Although it still remains speculative, the induction of oxidative stress by serum-free incubation may not be due to the facilitation of the generation of oxidative stress but due to an inadequate removal of oxidative stress under *ex vivo* conditions [29]. The observation that the incubation of isolated aortas at 4°C had no effect on the reactivity of the aorta may be consistent with the notion that the generation of oxidative stress was due to the normal cellular metabolism.

Oxidative stress has been reported to induce the inactivation of the released NO and inhibit NO production, thereby reducing the bioavailability of NO [24, 50, 51]. These effects of oxidative stress may be consistent with the suggestion that oxidative stress contributes to the pathogenesis of vascular diseases [24, 25, 52]. Therefore, the functional relevance of the increased relaxant response to PAR₂ stimulation seen after oxidative stress appears to be enigmatic. However, the enhancement of the PAR₂-mediated relaxation has also been observed after treatment with inflammatory cytokines [21–23] and in a diabetic model [53]. In a diabetic model, the relaxant response to acetylcholine was impaired [53]. The enhanced relaxant response to PAR₂ stimulation seen after oxidative stress may thus play some role in pathological conditions, such as inflammatory hyperemia. Furthermore, PAR₂ has been reported to induce cytokine production, leukocyte adhesion and exocytosis of a Weibel-Palade body in endothelial cells [9–12]. Therefore, the increased PAR₂ function may also be linked to the increased proinflammatory and procoagulant effect of PAR₂.

PAR₂-mediated endothelium-dependent relaxation has been reported to be preserved, while acetylcholine-induced relaxation was impaired in SHR [54]. The present study suggests the preserved basal expression of PAR₂ in SHR to be attributable to the increased level of *in vivo* oxidative stress. However, serum-free incubation further enhanced the relaxant response to trypsin in SHR. The present study also noted some sexual difference in the basal expression of PAR₂. The strips of male aortas showed a transient relaxation without serum-free incubation, as observed in SHR, although the role of oxidative stress in males still remains to be investigated. The sexual difference may also be linked to other factors such as sex hormones. Therefore, a separate investigation may thus be necessary to address the sexual difference in the regulation of the PAR₂ expression. Nevertheless, regardless of the difference in the basal expression of PAR₂, *in vitro* challenge with oxidative stress similarly enhanced the trypsin-induced relaxation in all specimens, thus

consistently suggesting the role of oxidative stress in up-regulating PAR₂ expression.

In conclusion, the observations of the present study suggest that oxidative stress upregulated the expression of PAR₂ and enhanced the responsiveness to PAR₂ stimulation in the endothelial cells of rat aortas. Moreover, the expression of PAR₂ in the endothelial cells of SHR aortas was also suggested to be preserved in an oxidative stress-dependent manner. The enhanced PAR₂ function seen after the exposure to oxidative stress may therefore be an underlying factor, at least in part, in the pathological effect of oxidative stress in vascular diseases.

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