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ORIGINAL PAPER

Alteration of discoidin domain receptor-2 expression: possible role in peroxynitrite-induced apoptosis in human cerebral vascular smooth muscle cells

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Abstract Peroxynitrite ($ONOO^-$), a product of nitric oxide and superoxide anion interaction, is a powerful and versatile oxidant. In this work, the human cerebral vascular smooth muscle cells (HCVSMCs) were cultured *in vitro* and subjected to different concentrations of $ONOO^-$. Cell viability and morphological changes were measured by methylthiazoletetrazolium (MTT) assay and Ho33342/PI double staining respectively. The apoptotic rate was assessed by flow cytometry. The alterations in the expression of DDR2 at both mRNA and protein levels were examined by real-time PCR and Western-blot simultaneously. Direct exposure of HCVSMCs to $ONOO^-$ was able to inhibit the cell proliferation, which was further revealed via the apoptotic pathway. $ONOO^-$ exerted a dual-effect on DDR2 expression at both mRNA and protein levels that depended on the concentrations of $ONOO^-$. Our results demonstrate for the first time that DDR2 gene may play an important role in $ONOO^-$-induced apoptosis in HCVSMCs.

Keywords Peroxynitrite, Discoidin domain receptor 2, Human cerebral vessel smooth muscle cell, Apoptosis

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Substantial evidence suggests that reactive nitrogen species (RNS) play pivotal roles in vascular-related diseases of diverse types, such as atherosclerosis, hypertension, and restenosis[1-3]. $ONOO^-$, a product of a reaction between nitric oxide and superoxide, is such a typical molecule of RNS. Under normal conditions, $ONOO^-$ generally keeps at nM level, whereas, it can rise up to μM level under abnormal circumstances, thereby implicating in a number of pathophysiological processes[4,5]. It has been demonstrated that $ONOO^-$ is able to diffuse freely across phospholipid membrane bilayers to target a wide variety of molecular targets, resulting in cell death via necrosis or apoptosis[1,6]. In *in vitro* studies, exposure of cells to $ONOO^-$ elicits responses that depend on concentration and cell types, including vascular smooth muscle cells (VSMCs)[7,8].

VSMCs, the major component that constitutes blood vessel wall, is the key determinant in vascular diseases relevant to different stimuli. Vascular diseases, in fact, are characterized by alterations of blood vessel structure determined mainly by VSMCs growth, which is now viewed as the result of the opposing effects of cell proliferation and apoptosis[9]. Apoptosis of VSMCs is a prominent feature of the vascular remodeling process that occurs in atherosclerosis, hypertension, and restenosis[10,11].

In response to injury, VSMCs enhance the collagen synthesis, which in turn regulate VSMC reactions to vascular repair via specific signaling pathway. Discoidin domain receptors (DDRs), the nonintegrin-type receptors for collagen, consist of two closely related types, i.e., DDR1, which mainly is mainly expressed in epithelial cells and, DDR2, which is highly expressed in mesenchymal cells[12]. It has been demonstrated

that DDRs and integrins act in coordinated manner to modulate cellular responses[13]. Evidence from DDR2-null mice and in vitro studies suggests that DDR2 can regulate cell proliferation and extracellular matrix remodeling mediated by matrix metalloproteinase (MMP) activities during normal development or pathological conditions[14]. Phosphorylation of DDR2 in cultured human VSMCs leads to decreased collagen biosynthesis and increased collagen breakdown that may contribute to the extracellular matrix remodeling[15]. Of note, Shyu *et al*. reported that certain stimuli, such as hyperbaric oxygen, hypoxia or mechanical stretch were able to alter the DDR2 expression in VSMCs[16]. Although DDR2 involves in regulation of collagen turnover mediated by VSMCs, little is known on its role in vascular system, especially in response to oxidative stress.

Previously, we found that $ONOO^-$ can induce apoptosis in canine and rat VSMCs[17,18]. However, the precise mechanism by which $ONOO^-$ elicits apoptosis in VSMCs is still unclear. Particularly, whether there exist a link between the expression of DDR2 and the action of PN is undefined in HVSMCs. Therefore, the present study was designed to determine whether HVSMCs undergo apoptosis following treatment with $ONOO^-$ and whether the process can occur in relationship with DDR2 activity.

Effects of $ONOO^-$ on cell viability of HCVSMCs

In this assay, OD values represented the absorption of formazan dissolved by DMSO at 570 nm. As evidenced by MTT assay, $ONOO^-$ treatment led to the apparent reduction of cell viability of the HCVSMCs at 24 h in a concentration-dependent manner (Figure 1). Use of decomposed $ONOO^-$ (see Materials and Methods) failed to exert any effects on cell viability (data not shown).

Effects of $ONOO^-$ on morphological changes in the HCVSMCs

Morphological changes were examined by Hochest 33342/PI double staining after direct exposure to different concentrations of $ONOO^-$ for 24 h. As illustrated in Figure 2, untreated cells exhibited blue intact nuclei, however, cells direct exposed to $ONOO^-$ showed blue or red nuclei. Cells with red staining were thought as necrosis. Cells without $ONOO^-$ treatment showed polygonal shape, but, cells treated with $ONOO^-$ became rounding-up, and had cytoplasmic contraction and chromatin condensation. Apoptotic bodies were present.

Flow cytometry analysis

The quantitative analysis of apoptotic cells was determined by Flow cytometry analysis. Compared with the apoptotic rate of untreated cells (5.84%), the rates exposed to 10, 50, 100 μM concentrations of $ONOO^-$ for 24 h were 5.2%, 11.6%, and 37.5%, respectively (Figure 3). The results further proved that $ONOO^-$ can induce apoptosis of the HCVSMCs.

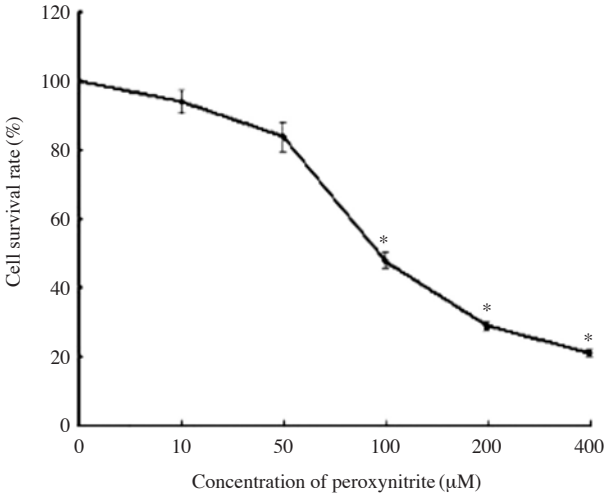

Figure 1. Effect of $ONOO^-$ on the viability of HCVSMCs. $ONOO^-$ significantly reduced cell viability in a concentration-dependent manner. $*P < 0.05$ compared with control group.

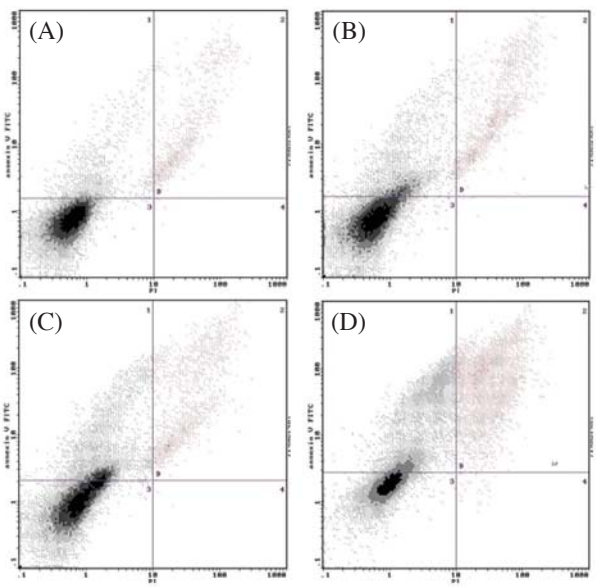

Figure 2. Morphological changes in HCVSMCs treated with $ONOO^-$ using Ho33342 and PI double staining under fluorescence microscopy (×200). A: control; B: 10 μM; C: 50 μM; D: 100 μM. Normal cells presented with blue intact nuclei; apoptotic cells showed blue nuclear fragments; and necrotic cells appeared red nuclei.

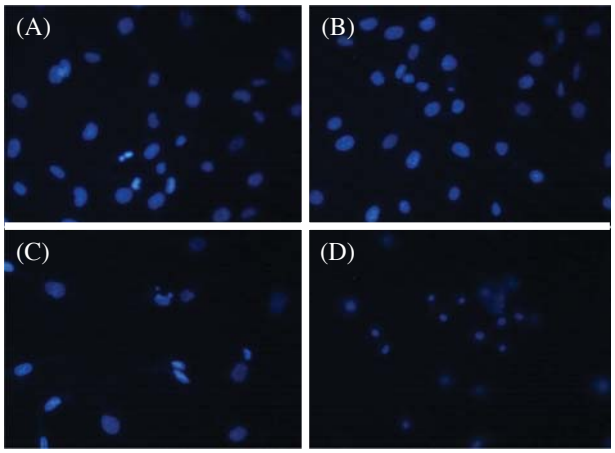

Figure 3. Apoptotic rates of the HCVSMCs treated with $ONOO^-$ using FCM. A: control; B: 10 μM; C: 50 μM; D: 100 μM. X-axis represents the density of propidium iodide (PI), Y-axis represents the density of Annexin V-FITC. Quadrant D1 represents the apoptotic rates of cells; quadrant D2 represents the necrosis of cells; quadrant D3 represents the normal cells; quadrant D4 represents the naked nucleus of cells. All experiments were conducted three times. Data were presented as mean ± SEM.

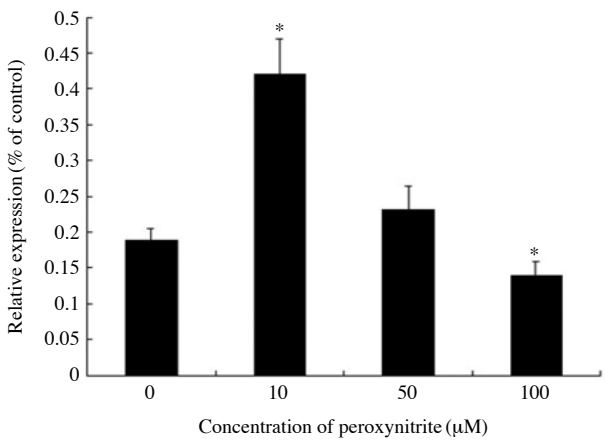

Figure 4. Effect of $ONOO^-$ on expression of DDR2 mRNA in HCVSMCs. The real time-PCR analysis showed that the amount of mRNA transcript for DDR2 was significantly higher in 10 μM $ONOO^-$ treated group, but, declined in 50 μM $ONOO^-$ treated group, and significantly lower in 100 μM $ONOO^-$ treated group compared with that in control group ($P < 0.05$).

Effects of $ONOO^-$ on mRNA expression of DDR2 in HCVSMCs by real time-PCR

In this work, the relative level of DDR2 mRNA transcript to control GAPDH was determined by real time-PCR. Compared with untreated cells, DDR2 mRNA expression in 10 μM $ONOO^-$ treated cells was up-regulated at 24 h, but, the expressions in other two experimental groups treated with $ONOO^-$ at concentrations of 50, 100 μM were down-regulated at 24 h Statistic analysis showed that the DDR2 mRNA expression was significantly higher in 10 μM $ONOO^-$ treated group, but, declined in 50 μM $ONOO^-$ treated group, and significantly lower in 100 μM $ONOO^-$ treated group compared with that in control group ($P < 0.05$).

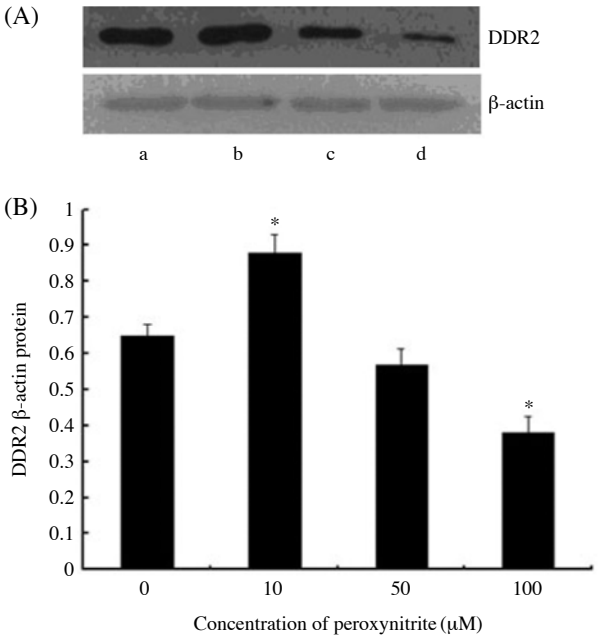

Figure 5. Effect of $ONOO^-$ on expression of DDR2 protein in HCVSMCs. A: Representative RT-PCR analysis of DDR2 in both control and cells exposed to $ONOO^-$. a: control; b: 10 μM; c: 50 μM; d: 100 μM. B: Protein levels were quantified by densitometric analysis and normalized for β-actin. The DDR2 protein expression was significantly higher in 10 μM $ONOO^-$ treated group, but, declined in 50 μM $ONOO^-$ treated group, and significantly lower in 100 μM $ONOO^-$ treated group compared with that in control group ($P < 0.05$)

Effects of $ONOO^-$ on protein expression of DDR2 in HCVSMCs by western-blot

In this study, the further determination of DDR2 protein expression in HCVSMCs in response to different concentrations was basically identical to DDR2 mRNA expressions. That is, compared with untreated cells, the activity of DDR2 in 10 μM $ONOO^-$ treated cells was up-regulated, whereas, the activities of DDR2 in the other two experimental groups at concentrations of 50, 100 μM of $ONOO^-$ at 24 h were gradually down-regulated (Figure 5A). Image J software was used to analyze the relative photodensity, with β-actin as standard (Figure 5 B). Statistic analysis showed that DDR2 protein expression was significantly higher 10 μM $ONOO^-$ treated group, but, decreased in 50 μM $ONOO^-$ treated

group, and significantly lower in 100 μM $ONOO^-$ treated group compared with that in control group ($P < 0.05$).

Discussion

It has been confirmed that the pathogenesis of vascular diseases involves a perturbation of the balance between cell proliferation and cell death triggered by many mediators, including RNS[19]. In the current study, we found that $ONOO^-$ reduced cell surviving rate in a concentration- and time-dependent manner as evidenced by MTT assay, suggesting that $ONOO^-$ was able to inhibit the growth of HVSMCs. Meanwhile, the exposure of HVSMCs to $ONOO^-$ resulted in cellular rounding-up, cytoplasmic contraction, chromatin condensation and, particularly, the apoptotic body using Ho.33342 and PI double staining, which were in accordance with typical morphological characteristics of cells experienced apoptosis. Moreover, the apoptotic rates were increased when cells exposed to these drugs as confirmed by flow cytometry. This further revealed the occurrence of apoptosis, rather than necrosis, by $ONOO^-$. These data were similar with our previous findings in rat and canine VSMCs[17,18].

It has been documented that apoptosis of VSMC, which is triggered by various agents, plays a key role in the pathogenesis in a variety of vascular diseases[20]. However, the precise mechanisms underlying the inhibitory effects of $ONOO^-$ on VSMCs are largely undetected. Recently, Shyu *et al*. found that up-regulation of DDR2 expression significantly increased the migration and proliferation of VSMCs[16]. In this work, we focused on investigating whether or not DDR2 is a key determinant in $ONOO^-$-elicited apoptosis of HVSMCs. Interestingly, we demonstrated that, for the first time, expression of DDR2 mRNA increased as HCVSMCs were subjected to lower concentration of $ONOO^-$, whereas, the expression of DDR2 mRNA decreased as HCVSMCs were subjected to higher concentration of $ONOO^-$. The similar tendency was found in DDR2 expression at protein level in response to $ONOO^-$. This indicates that $ONOO^-$ exerts a dual-effect on DDR2 expression in HCVSMCs, which depends on the concentrations of $ONOO^-$. The possible explanation for the difference in DDR2 reactivity might be related to the adaptive reaction to environmental stress and cell type used. That is, the initial up-regulation of DDR2 expression might be the cellular compensation for the damage by $ONOO^-$ and, as the stress enhanced, the damage exceeds compensatory ability of VSMC, leading to down-regulation of DDR2 expression.

Overall, our findings suggested that DDR2 is likely a key determinant that participates in $ONOO^-$-induced apoptosis of HVSMCs. Currently, two major apoptotic mechanisms, i.e., mitochondrial pathway and death receptor pathway, both of which result in the activation of caspase cascades via a series of interactions, have been well established[21]. This poses a question that whether or not DDR2 involved in these two classical pathways in $ONOO^-$-induced apoptosis of HCVSMCs remains to be studied further.

In conclusion, the present study demonstrates that exogenous $ONOO^-$ can elicit apoptosis in cultured HVSMCs. This $ONOO^-$-induced apoptosis appears to involve in activation of DDR2. The precise mechanism responsible for $ONOO^-$-induced apoptosis in HVSMCs needs to be extensively investigated, which may in turn lead to effective therapeutic strategies by the intervention of endogenous $ONOO^-$ production.

Materials & Methods

Materials

$ONOO^-$ was purchased from Cayman CHEMICAL (Ann, Arbor, MI, USA, Cat. 81565) and deep frozen to −80°C before use, as the product is heat and light sensitive. Activity decreases approximately 2% per day at −20°C. The HCVSMC line was purchased from the ScienCell Research Laboratories (USA). Smooth muscle cell medium for definite purpose, fetal bovine serum, and smooth muscle cell growing factors were also purchased from the ScienCell Research Laboratories (USA). Rabbit-anti-DDR2 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (USA). The other agents were purchased from Sigma (St. Louis, MO, USA).

Cell culture

The HCVSMCs were cultured in smooth muscle cell medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2. Cells were regularly sub-cultured with 0.25% trypsin. Subsequently, cells were seeded into Petri dish, 24 or 96-well plate for different experiments.

Cell viability assessment

The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay was used to assess the cell viability. Cells (1×10^4/per well) were seeded into 96-well plate containing 100 μL culture medium.

Twenty-four hours before $ONOO^-$ treatment, the medium was replaced with 1% serum medium. $ONOO^-$ was diluted in phosphate-buffered saline (PBS; pH 8.3)

and was directly added to the culture medium at final concentrations of 10, 50, 100, 200, and 400 μM for 24 h incubation. Control sample was treated with PBS (pH 8.3) only. The effect of decomposed $ONOO^-$ was also tested after the solution of $ONOO^-$ was kept in PBS (pH 7.2) at 37°C for 30 min. At the indicated time points, MTT was added to reach a final concentration of 0.5 mg/mL. After 4 h of incubation at 37°C, the medium was removed and the precipitate in each well was dissolved in dimethylsulfoxide. The plates were incubated for another 20 min in the dark. The optical density (OD) values were measured at 570 nm using an ELISA reader (Multiskan MK3, Finland).

Morphological observation

Morphological changes of HCVSMCs in response to different concentrations of $ONOO^-$ for 24 h of incubation were detected by Ho33342 and PI double staining. Briefly, cells (5×10^4/per well) were seeded into 24-well dishes containing 1 mL culture medium. After 24 h of incubation, the medium was removed and refilled with new serum-free medium containing, 10, 50, 100 μM concentrations of $ONOO^-$ for 24 h incubation. The cells were washed twice by PBS, fixing with 95% alcohol for 10 min, and then stained by Ho33342 (10 μg/mL) and PI (50 μg/mL) 37°C for 30 min. After washed twice with PBS, the morphological changes were examined by fluorescence microscope. Nuclear fragmentation and/or marked condensation of chromatin, apoptotic bodies were thought to be typical features of apoptotic cells.

Flow cytometry analysis

Cell apoptotic progression was assessed by flow cytometry using Annexin V-FITC and propidium iodide (PI) staining. Briefly, cells (5×10^4/mL) were seeded into 6-well dishes and were treated with $ONOO^-$ for various time points. After treatment, cells were trypsinized, washed with phosphate-buffered saline (PBS). After washing, cells (1×10^7/mL) were resuspended in binding buffer (200 μL) containing Annexin V-FITC (5 μL, 20 μg/mL) and PI (10 μL, 20 μg/mL) for 15 min at room temperature, and then added Binding Buffer (300 μL) before analysis with a FACScan (BectonDickinson, Mountain View, CA, USA) using CellQuest-software (BD Biosciences, San Jose, CA, USA).

An isolation of total RNA and and real time-PCR

After treated with $ONOO^-$ at concentrations of 0 (control), 10, 50, and 100 μM for 24 h, total RNA was isolated from HCVSMCs using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The mRNA expression of DDR2 gene was examined using real time-PCR with M-MuL V reverse transcriptase in the presence of random hexamer primer. Using SYBR Green PCR assay, each 25 μL PCR reaction mixture contained 12.5 μL 2 × SYBR Green PCR Master Mix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 2 μL template, and 9.5 μL sterilized distilled water. Each treatment contained three samples and each PCR was carried out in triplicate. The conditions of PCR were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 61°C for 15 s and 72°C for 30 s followed by dissociation at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. All data were analyzed using the Eppendorf realplex2. The quantity of target cDNA or GAPDH PCR product was calculated using the corresponding standard curve and the amount of target cDNAs in a given sample was normalized with that of GAPDH cDNA. The normalized quantity of target cDNA in the RNA sample from untreated control cells was used as a calibrator. PCR primers for the genes of DDR2 and GAPDH were as follows.

DDR2: F: 5′-GACCGCATCAGGAATTTCACTAC-3′;
R: 5′-GGATCCGAGTGTTGCTGTCATC-3′.
GAPDH: F: 5′ GTGGGGCGCCCCAGGCACCA 3′
R 5′ CTCCTTAATGTCACGCACGATTT 3′

Protein extraction and western blot analysis

The HCVSMCs were harvested and lysed with a RIPA protein lysis buffer for 30 min on ice. The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 × g for 15 min at 4°C. The supernatant was kept at −80°C until use. The Bradford method was used to determine the protein concentration of the supernatant. Samples (40 μg of total protein each) were boiled at 95°C for 5 min and loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 5% stacking gel and 15% separating gel), followed by a separation at 80 V for about 2 h and subsequent transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% defatted milk for 1 h at room temperature, incubated with the first antibodies (DDR2 1 : 1000, Rabbit anti-Human; β-actin, 1 : 2000, mouse anti-human), and diluted in 5% defatted milk/Tris-buffered saline-Tween (TBST) overnight at 4°C. After washing with TBST, the membrane was incubated with a secondary antibody against mouse IgG accordingly and the signals were visualized using ECL. The actin and DDR2 bands were visualized at apparent molecular weights of 43 kDa and 116 kDa, respectively. Relative OD ratio was calculated with NIH software Image J by comparing to β-actin

from three experiments.

Statistic analysis

Our data were presented as mean ± standard error of the mean (SEM). Statistical calculations were performed using SPSS11.5 software package. One-way analysis of variance (ANOVA) was applied to analyze these data. *P* values of less than 0.05 were considered significant.

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