Neutrophil extracellular traps accelerate vascular smooth muscle cell proliferation via Akt/CDKN1b/TK1 accompanying with the occurrence of hypertension

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Objective: Neutrophil extracellular traps (NETs) can trigger pathological changes in vascular cells or vessel wall components, which are vascular pathological changes of hypertension. Therefore, we hypothesized that NETs would be associated with the occurrence of hypertension.

Methods: To evaluate the relationship between NETs and hypertension, we evaluated both the NETs formation in spontaneously hypertensive rats (SHRs) and the blood pressure of mice injected phorbol-12-myristate-13-acetate (PMA) via the tail vein to induce NETs formation in arterial wall. Meanwhile, proliferation and cell cycle of vascular smooth muscle cells (VSMCs), which were co-cultured with NETs were assessed. In addition, the role of exosomes from VSMCs co-cultured with NETs on proliferation signaling delivery was assessed.

Results: Formation of NETs increased in the arteries of SHR. PMA resulted in up-regulation expression of citrullinated Histone H3 (cit Histone H3, a NETs marker) in the arteries of mice accompanied with increasing of blood pressure. NET treatment significantly increased VSMCs count and accelerated G1/S transition in vitro. Cyclin-dependent kinase inhibitor 1b (CDKN1b) was down-regulated and Thymidine kinase 1 (TK1) was up-regulated in VSMCs. Exosomes from VSMCs co-cultured with NETs significantly accelerated the proliferation of VSMCs. TK1 was up-regulated in the exosomes from VSMCs co-cultured with NETs and in both the arterial wall and serum of mice with PMA.

Conclusion: NETs promote VSMCs proliferation via Akt/CDKN1b/TK1 and is related to hypertension development. Exosomes from VSMCs co-cultured with NETs participate in transferring the proliferation signal. These results support the role of NETs in the development of hypertension.

Keywords: exosome, hypertension, neutrophil extracellular traps, thymidine kinase 1, vascular smooth muscle cells

Abbreviations: CDK, cyclin-dependent kinases; CDKN1b, cyclin-dependent kinase inhibitor 1b; cit Histone H3, citrullinated histone H3; DAVID, the Database for Annotation Visualization and Integrated Discovery; DEGs, differentially expressed genes; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HCD, higher energy collisional dissociation; MBP, mean blood pressure; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PAD4, peptidyl arginine deiminase 4; PMA, phorbol-12-myristate-13-acetate; PPI, protein–protein interaction; SHR, spontaneously hypertensive rat; TEM, transmission electron microscopy; TK1, thymidine kinase 1; VSMCs, vascular smooth muscle cells; WKY, Wistar–Kyoto rat

INTRODUCTION

Essential hypertension is a major public health issue [1,2]. The cause is complex, involving interactions among genetic and environmental factors [3]. The pathogenesis of essential hypertension is not fully understood [3,4].

Inflammation is closely related to the occurrence and development of hypertension [5–7]. However, the mechanism underlying inflammation-induced hypertension is unclear. Chronic inflammation occurs in a state of low-grade inflammation after an acute inflammatory process when the initiating stimulus is not removed or if the resolution program is disturbed [8]. Chronic inflammation can last for a long time, causing tissue damage, fibrosis, and irreversible organ dysfunction [9,10]. Neutrophils have been shown to play a...
significant role both in acute and chronic inflammation. Neutrophils are the first line of defense against inflammation. They are recruited to inflammatory sites to eliminate harmful substances. In response to chronic and low-grade inflammatory reactions, neutrophils can form neutrophil extracellular traps (NETs) that cause a particular form of cell death, NETosis, and continue to capture and kill microorganisms, preventing the spread of infection. NETs are formed by the chromatin reticulum, in which a variety of proteins are embedded, such as citrullinated histone H3 (cit Histone H3) and myeloperoxidase (MPO) [11–15].

NETs have been detected in human vascular tissues and are related to vascular wall lesions, such as atherosclerosis [16–20]. Furthermore, NETs can participate in the development of pulmonary hypertension by promoting angiogenesis [20]. Proteins involved in NETs formation, such as peptidyl arginine deiminase 4 (PAD4), can cause fibrosis [21]. Fibrosis is a pathological change in vascular remodeling related to the occurrence and development of hypertension [22]. Moreover, NETs could promote gastric cancer cell proliferation, invasion, migration and epithelial–mesenchymal transition dependent on TGF-β signaling [23]. Furthermore, it has been proved that TNF-α up-regulated neutrophil elastase in vascular smooth muscle cells (VSMCs), which promoted VSMC migration, proliferation and inflammation [24] and mediates the formation of NETs [25]. VSMC proliferation is one of the key vascular pathological changes occurring during the development of hypertension [26].

On the basis of these studies, we hypothesize that NET formation in arteries probably would be related with the development of hypertension by promoting VSMC proliferation. Therefore, the aim of the study is to evaluate the association between arterial NET formation and increasing blood pressure and explore the role of NETs on the cellular and molecular mechanisms of hypertension.

METHODS

Details on the protocol used in this study are shown in the flowchart in Fig. 1.

Animals

Spontaneously hypertensive rat (SHR) and Wistar–Kyoto (WKY) (male, 6 weeks old) were raised to 20 weeks of age to assess the formation of NETs in the arteries under high blood pressure in vivo.

In addition, NETs formation in the arteries of BALB/c mice (male, 10–12 weeks old) was induced by phorbol-12-myristate-13-acetate (PMA; Sigma, St Louis, Missouri, USA) [20,27]. PMA (200 μl of 100 nmol/l) was injected in the tail vein once every 3 days for 3 months to induce NET formation.

All animals were obtained from the Animal Center of China Medical University and kept under controlled conditions (light : dark cycle of 12 : 12, starting at 0600 h; 22–23°C; 45–50% relative humidity).

Blood pressure evaluation

SBP was evaluated in mice at month 0 (basal) and month 3 after PMA injection, and in rats at week 6 and week 20 using a standard tail cuff [28,29].

Fang et al.

Immunochemistry assay

Sections of superior mesenteric artery of SHR and WKY were blocked with 0.3% hydrogen peroxide, followed by incubation with 5% normal goat serum and then with a rabbit polyclonal anticit Histone H3 antibody at 4°C overnight. Next, the sections were incubated with the biotinylated secondary antibody. Visualization was performed via the diaminobenzidine reaction and hematoxylin staining. The staining intensities were determined by measurement of the integrated optical density by light microscopy using a computer-based Image-Pro Morphometric System by two independent observers in a double-blind manner.

Neutrophil isolation and neutrophil extracellular trap induction

Neutrophils were isolated from whole blood of Wistar rat and induced by PMA to form NETs in vitro. Male 10-week-old Wistar rat was sacrificed and 4 ml whole blood per rat was collected from the left ventricle in EDTA-treated collection tubes. Mature neutrophils were purified by centrifugation for 30 min at 1500 g on a discontinuous Percoll gradient consisting of 52% (v/v), 69% (v/v), and 78% (v/v) Percoll in PBS. Mature neutrophils were recovered from the interphase between 69 and 78% Percoll and were pelleted. Remaining erythrocytes were lysed using red blood cell lysis buffer. Neutrophils were washed in PBS and pelleted again by centrifugation for 10 min at 300 g to remove the remaining lysis buffer. NETs formation was induced from neutrophils by overnight stimulation with 100 nmol/l PMA.

Cell culture and treatments

Rat VSMCs were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Waltham, Massachusetts, USA) with 10% fetal bovine serum (FBS) (Gibco). VSMCs were cultured with NETs for 24 h (per 1 × 10⁶ VSMCs: NETs induced from 1 × 10⁶ neutrophils). Meanwhile, in another group that VSMCs were cultured with Ang II (10⁻7 mol/l; Sigma), and this group was performed as a positive control.

Cell proliferation

VSMC proliferation was evaluated using the CellTiter 96 One Solution Proliferation Assay (MTS, G3580; Promega, Madison, Wisconsin, USA) in accordance with the manufacturer’s guidelines. Cells were seeded in a 96-well tissue culture plate (5 × 10⁴ cells/well). OD values were read at 490 nm.

Cell cycle analysis

Cells were seeded in a six-well tissue culture plate (5 × 10⁵ cells/well). For G0/G1 synchronization, cells were synchronized by serum deprivation for 12 h. After treatment, the cells were collected and washed. Cells were resuspended in a mixture of RNaseA solution (50 μl) and propidium iodide (PI; 450 μl) and incubated for 30 min in the dark. The DNA content was detected by flow cytometry. The percentages of cells in the G0/G1 phase, S phase, and G2/M phase were analyzed.
**Cell apoptosis assays**

Cells were seeded in a six-well tissue culture plate (5 x 10^5 cells/well). After treatment, cells were collected and washed. Cells were resuspended in 500 µl of binding buffer premixed with 5 µl of Annexin V-FITC and 5 µl of PI and incubated for 15 min at room temperature in the dark. Cells were analyzed with flow cytometry (BD Biosciences, Baltimore, Maryland, USA) within 1 h.

**Label-free proteomic analysis**

Proteins were extracted using SDT buffer [4% SDS, 100 mmol/l dithiothreitol (DTT), 150 mmol/l Tris–HCl, pH 8.0] and quantified. Approximately, 200 µg of proteins from each sample was supplemented with 30 µl of SDT buffer. After washing with detergent, DTT and other low-molecular-weight components were removed, and cysteine residues were blocked. The protein suspensions were
digested with 4 μg of trypsin (Promega) in 40 μl of 25 mMol/l NH₄HCO₃ buffer, and the resulting peptides were collected. The peptides in each sample were desalted on C₁₈ Cartridges (Empore SPE Cartridges C₁₈ (standard density), bed I.D. 7 mm, volume 3 ml; Sigma, St Louis, Missouri, USA), concentrated by vacuum centrifugation, and reconstituted in 40 μl of 0.1% (v/v) formic acid. The OD²₈₀ values for the resulting peptides were determined. Each fraction was injected into the nano liquid chromatography/tandem mass spectrometry (nanoLC-MS/MS) system. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C₁₈; Waltham, Massachusetts, USA) connected to the C₁₈-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% formic acid) and buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nl/min. The 120 min LC gradient was 0–55% buffer B for 110 min, 55–100% buffer B for 5 min, and 100% buffer B for 5 min.

MS data were acquired using a data-dependent top 10 method, in which the most abundant precursor ions were dynamically chosen from the survey scan (300–1800 m/z) for higher energy collisional dissociation (HCD) fragmentation. The automatic gain control target was set to 3e6, and the maximum inject time was set to 10 ms. The dynamic exclusion duration was 40 s. Survey scans were acquired at a resolution of 70 000 at m/z 200 and resolution for HCD spectra was set to 17 500 at m/z 200, and the isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.15. The instrument was run with peptide recognition mode enabled. The MS data were analyzed using MaxQuant version 1.5.3.17 (Max Planck Institute of Biochemistry, Martinsried, Germany).

Bioinformatic analysis
A heatmap was generated to visualize relative protein expression data. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) was used for a Gene Ontology (GO) analysis. The heatmap and GO annotation results were plotted using R scripts. Protein–protein interaction (PPI) information was retrieved using STRING (version 11.5). The results were downloaded in the XGMML format and imported into Cytoscape5 for visualization and further hub genes analysis.

TK1 gene silencing
TK1 expression was inhibited by using Hu6-MSC-CMV-GFP-SV40-Neomycin vectors and two siRNA sequences targeting TK1 (target sequence of siRNA sequence 1: GCGGACAAG-TACCACCTGTGT and target sequence of siRNA sequence 2: GTGCTTTCGAGAAGCTTCCTA). In parallel, an unrelated siRNA was designed as a negative control. Plasmid transfection was performed using Lipofectamine 3000 (L3000015; Invitrogen, Carlsbad, California, USA).

Exosome isolation and purification
Using sequential ultracentrifugation, exosomes were collected from the culture medium of VSMCs, VSMCs cultured with NETs, and TK1 silencing VSMCs, respectively. Briefly, the cell culture medium was centrifuged at 200 g for 20 min and 2000 g for 20 min and the supernatants were filtered by a 0.22 μm filter. The filtrates were collected for centrifugation at 10 000 g for 1 h and 100 000 g for 4 h. The final pellet containing exosomes was resuspended in PBS and used for transmission electron microscopy (TEM), western blotting of CD9 and Alix (two exosome markers), and an exosome diameter analysis. Then the exosomes were subsequently added into VSMC culture medium to observe the role of the exosomes.

Transmission electron microscopy
Exosomes were fixed with 2% glutaraldehyde in 0.1 mol/l PBS (pH 7.4). Fixed samples were placed on 100 mesh carbon and formvar-coated nickel grids for about 30 min. After samples were washed with several drops of ultra-pure water, 2% uranyl acetate was pipetted on the grids and incubated for 7 min. Excess solution was removed. The grid was washed twice and dried. Images were obtained by TEM (80 kV, 60 000×, H-7650; HITACHI, Tokyo, Japan).

Laser particle size analyzer
The particle size was assessed using the Laser Particle Size and Zeta Potential Analyzer (Nano ZS90; Malvern Instruments, Malvern, UK). Nanoparticles were diluted (1:10) with deionized water prior to analyses.

Western blot analysis
Lysates from exosomes, cells, and arterial tissues were prepared with RIPA lysis buffer. Protein lysate and serum of mice injected PMA, SHR and WKY were resolved by 10% SDS–PAGE and electrotransferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk for 2 h at room temperature and incubated with primary antibodies at 4 °C overnight [anti-CD9 (1:1000, Abcam, ab92726), anti-Alix (1:1000, Abcam, ab275377), anti-TK1 (1:500, Proteintech, 15691–1-AP), anti-CDKN1b (1:500, Proteintech, 25614–1-AP), anti-TOP2a (1:500, Abcam, ab52934), anti-ci-trullinated histone H3 (1:1000, Abcam, ab5103), anti-MPO (1:1000, Abcam, ab28670) and GAPDH (1:500, Proteintech, 10494–1-AP)]. After incubation with the horseradish peroxidase-conjugated secondary antibody and washing, blots were developed with chemiluminescent reagents (WBKIS0100; Millipore, Billerica, Massachusetts, USA).

Statistical analysis
Differentially expressed proteins between VSMCs and VSMCs co-cultured with NETs were identified based on a fold change greater than 2 and P less than 0.05 using the Wilcoxon rank sum test. Gene ontology enrichment was determined based on the Fisher’s exact test. Benjamini–Hochberg correction was applied for multiple testing. Only functional categories with P values of less than 0.05 were considered significant.

Statistical analyses were performed using SPSS 22.0 (IBM, Armonk, New York, USA). Results are presented as means ± SEM. Differences between two groups were analyzed with Student’s t tests. Differences among multiple groups were analyzed with one-way ANOVA. For all statistical comparisons, P less than 0.05 was considered significant.
RESULTS

Neutrophil extracellular trap formation increased in arterial wall with hypertension in spontaneously hypertensive rat model

To explore the close relationship between the formation of NETs in arterial wall and the occurrence of hypertension, we assessed the NETs formation in the arteries of the SHR model. First, blood pressure [including SBP, DBP, and mean blood pressure (MBP)] was measured at 20 weeks in SHR. WKY rats were as control. At 20 weeks, blood pressure was higher in SHR than in WKY rats (SHR vs. WKY: \( P_{SBP} = 0.002, P_{DBP} = 0.0004, P_{MBP} = 0.0005, n = 6 \)) (Fig. 2a) Moreover, cit Histone H3 staining, a marker of NET formation was detected (Fig. 2b). The expression of cit Histone H3 in the superior mesenteric artery was higher in SHR than that in WKY rats (SHR vs. WKY: \( P = 0.0022, n = 6 \)) (Fig. 2c). The increase in blood pressure in SHR is caused by genetic factors as we detected an increase formation of NETs in their arteries of SHR.

The formation of neutrophil extracellular traps in the artery was accompanied by hypertension

To explore the role of NETs on blood pressure in vivo, NETosis was induced in vessels by injecting of PMA in the tail vein of mice. Mice injected with physiological saline were used as the control. After 3 months of sustained blood vessels low-dose inflammation, western blotting revealed that the NETs markers MPO and cit Histone H3 were upregulated in the superior mesenteric artery of mice with NETs formation (Fig. 2d). The expression level of MPO (NETs group vs. control group: \( P = 0.0107, n = 20 \); control: \( n = 13 \)), cit Histone H3 (NETs vs. control: \( P = 0.0058, n = 20 \); control: \( n = 13 \)) in the superior mesenteric artery was elevated in mice injected of PMA (Fig. 2e and f). When blood pressure was measured. The SBP, DBP, and MBP were significantly higher than those in the group receiving physiological saline as control (NETs group vs. control group: \( P_{SBP} = 0.0004; P_{DBP} < 0.0001; P_{MBP} < 0.0001, n = 20 \); control: \( n = 20 \)) (Fig. 2g). NET formation in PMA-injected mice was accompanied by an increase in blood pressure, indicating that NET formation is related to increasing blood pressure.

Neutrophil extracellular traps promote vascular smooth muscle cell proliferation and expedite the G1 to S phase transition

To study the effect of NETs on blood pressure, we further explored their effect of NETs on VSMC proliferation, cell cycle progression, and apoptosis. First, neutrophils were
extracted and then NETs formation was induced in vitro using PMA. The formation of NETs was confirmed via immunofluorescence staining of Granulocyte and cit Histone H3 (Fig. 3a). Then, VSMCs were cultured with NETs. NETs significantly increased VSMC proliferation from day 1 to 5 compared with proliferation rate in the control group (NETs group vs. control group: the first day $P = 0.007$, the second day $P = 0.049$, the third day $P = 0.006$, the fourth day $P = 0.0089$, the fifth day $P = 0.0008$, $n = 6$) (Fig. 3b). AngII is known to promote VSMC proliferation [18]. As expected, we found that treatment with AngII treatment resulted in a significantly higher rate of proliferation from days 3 to 5 than the rates in the control group (Fig. 3b and c).

The rapid proliferation of VSMCs induced by NETs might be caused by the acceleration of cell cycle progression or inhibition of apoptosis. We found that NETs decreased the proportion of cells in the G1 phase and increased that in the S phase. Similarly, AngII decreased the proportion of VSMCs in G1 and increased that in the S phase (NETs group vs. control group, $P_{G1\ phase} = 0.0066$ and $P_{S\ phase} = 0.0049$, $n = 4$) (Fig. 3d and e). Compared with the control, there were no differences in the rate of apoptosis relative to controls between NETs-treated and AngII-treated VSMCs at various apoptosis stages ($n = 4$) (Fig. 3f and g). These findings suggest that NETs facilitate VSMC proliferation, and this effect is mediated, at least in part, by the promotion of the G1/S transition and is not related to a reduction in apoptosis.

**Proteomic and bioinformatics analyses and Immunological validation**

A label-free proteomic analysis was used to identify differentially expressed proteins between VSMCs and VSMCs stimulated with NETs to explore the molecular mechanisms underlying the effect of NETs on VSMC proliferation. A hierarchical clustering analysis showed that 141 proteins were differentially expressed between NET-treated VSMCs and VSMCs, including 25 up-regulated proteins in NET-treated VSMCs, 18 down-regulated proteins in NET-treated VSMCs (Fig. 4a), 44 proteins expressed specifically in NET-treated VSMCs, and 54 proteins expressed specifically in VSMCs (Fig. 4b). Of note, CDKN1b, which inactivates
cyclin-dependent kinases (CDK) when bound and induces cell cycle arrest at the G1/S checkpoint, was only detected in the control group and not in VSMCs cultured with NETs. Furthermore, a pathway enrichment analysis showed that the PI3K-Akt pathway was enriched in VSMCs after NET stimulation. The activation of the PI3K-Akt pathway is known to promote cell growth via the inhibition of CDKN1b (Fig. 4c) [30]. To identify candidate differentially expressed proteins with critical roles in VSMCs after NETs stimulation, all significantly upregulated differentially expressed proteins were used for a PPI network analysis, and 13 hub genes were identified and used to construct a co-expression network (Fig. 4d). In the co-expression network, TOP2a was a central component. TK1, expressed specifically in VSMCs cultured with NETs, was a neighboring protein that directly interacted with and was controlled by TOP2a. TK1 is related to cell proliferation. It catalyzes the addition of a gamma-phosphate group to thymidine to create dTMP, which is the first step in the biosynthesis of dTTP, required for DNA replication. On the basis of our bioinformatic analysis, the PI3K/Akt/CDKN1b-signaling axis was activated by NETs in VSMCs based on our bioinformatic analysis. TOP2a expression increased and positively regulated TK1 after the inhibition of CDKN1b.

To validate the results of the proteome analysis, CDKN1b, TOP2a, and TK1 were analyzed using western blotting. Levels of CDKN1b were lower and levels of TOP2a and TK1 were higher in the NETs-treated group than in the control group, CDKN1b (NETs group vs. control group: \( P = 0.0276, n = 4 \)), TOP2a (NETs group vs. control group: \( P = 0.0276, n = 4 \)), and TK1 (NETs group vs. control group: \( P = 0.015, n = 4 \)).
Neutrophil extracellular traps promote vascular smooth muscle cells proliferation via TK1

RNAi was used to knockdown TK1 in VSMCs. Two TK1-specific RNAi fragments (shTK1–1 and shTK1–2) were transfected into VSMCs and the downregulation of TK1 was confirmed by western blotting (shTK1–1 vs. NC: P = 0.0173, n = 3, shTK1–2 vs. NC: P = 0.0398, n = 3) (Fig. 5a and b). After TK1 was knocked down, cell proliferation decreased (the fourth day: shTK1–1 vs. NC: P = 0.00161, n = 4, the fifth day: shTK1–1 vs. NC: P = 0.0008, n = 4, shTK1–2 vs. NC: P = 0.0089, n = 4) (Fig. 5c and d). Furthermore, the NET-induced increase in VSMC proliferation was blocked by TK1 knockdown in VSMCs (the first day: shTK1–1+NETs vs. NC+NETs, P < 0.0001, shTK1–2+NETs vs. NC+NETs, P = 0.0005; the second day: shTK1–1+NETs vs. NC+NETs, P = 0.0102; the third day: shTK1–1+NETs vs. NC+NETs, P = 0.0469, shTK1–2+NETs vs. NC+NETs, P = 0.0408; the fourth day: shTK1–1+NETs vs. NC+NETs, P = 0.0166; the fifth day: shTK1–1+NETs vs. NC+NETs, P = 0.0002, shTK1–2+NETs vs. NC+NETs, P < 0.0001, n = 4) (Fig. 5a and d).

Exosomes derived from vascular smooth muscle cells proliferation-neutrophil extracellular traps cultured promote vascular smooth muscle cell proliferation and cell cycle progression by transferring TK1

We hypothesized that exosomes might transmit proliferation signals from VSMCs in contact with NETs to those not in contact [31–33]. To evaluate this hypothesis, exosomes derived from the VSMC culture medium were isolated by ultracentrifugation and identified by TEM, a laser particle size analyzer, and western blotting. TEM revealed that exosomes had a cup-shaped morphology (Fig. S1A, http://links.lww.com/HJH/C3). As determined using a laser particle size analyzer, most particles were approximately 50–150 nm in diameter with a peak at around 100 nm (Fig. S1B, http://links.lww.com/HJH/C3). The exosomal protein markers Alix and CD9 were detected in these fractions (Fig. S1C, http://links.lww.com/HJH/C3).

Cell proliferation, cell cycle progression, and apoptosis in VSMCs treated with exosomes derived from VSMCs (exosome group) and VSMCs treated with exosomes derived from NETs-treated VSMCs (NETs exosome group) were evaluated. VSMCs cultured with NETs exosomes showed significantly greater proliferation than that in the exosome group (NETs exosome group vs. exosome group: the third day P = 0.0016, the fifth day P = 0.0101, n = 4; exosome group vs. control group: the fourth day P = 0.0008, the fifth day P = 0.006, n = 4) (Fig. 6a and b). The exosome group showed increased proliferation compared with the control group.

Furthermore, a cell cycle analysis using flow cytometry revealed that the exosome group had a lower proportion of cells in the G1 phase and higher proportion of cells in the S phase than those in the control group (exosome group vs. control group: PG1 spec phase = 0.0291, PS spec phase = 0.0133, n = 4; NETs exosome group vs. exosome group: PG1 spec phase = 0.001, PS spec phase < 0.0001, n = 4) (Fig. 6c and d). The proportion of cells in the G1 phase was lower and the proportion of cells in the S phase was higher in the NETs exosome group than those in the exosome group. These findings indicated that NETs promoted the G1/S transition (Fig. 6c and d).

There were no differences in the rate of apoptosis at various stages among the three groups (n = 4) (Figs. S2A and S2B, http://links.lww.com/HJH/C4). These findings suggest that exosomes from VSMCs facilitated VSMC proliferation and promoted the G1/S transition, without affecting apoptosis. Moreover, exosomes derived from NET-treated VSMCs had greater effects than those from VSMCs. After NET stimulation, TK1 was up-regulated in VSMC-derived exosomes (exosomes derived from NETs treated VSMCs vs. exosomes derived from VSMCs: P = 0.0086, n = 4) (Fig. 6e and f). We hypothesized that the exosome-induced increases in VSMC proliferation were mediated by TK1 signaling. To explore this, exosomes from VSMCs in which TK1 was knocked down were isolated. With the knockdown of TK1 in VSMCs, exosomal TK1 was concurrently downregulated (shTK1–1 vs. NC: P = 0.0083, n = 3, shTK1–2 vs. NC: P = 0.0125, n = 3) (Fig. 6g and h). In VSMCs cultured with these exosomes, the rate of proliferation was significantly lower than that of VSMCs cultured with VSMC-derived exosomes without TK1 knockdown (the fourth day: VSMCs + shTK1–1 vs. VSMCs + NC exosomes, P = 0.0232; the fifth day: VSMCs + shTK1–1 vs. VSMCs + NC exosomes, P = 0.0044, VSMCs + shTK1–2 vs. VSMCs + NC exosomes, P = 0.0002, n = 4) (Fig. 6i and j).

TK1 was up-regulated in the artery and serum of mice with phorbol-12-myristate-13-acetate-induced neutrophil extracellular traps formation

Our results showed that NETs promote cell proliferation by up-regulating the expression of TK1 in VSMCs in vitro. We then evaluated whether NETs induce the upregulation of TK1 in the arteries, indicating a NETs-induced increase in VSMC proliferation. TK1 expression was evaluated in the arteries of mice injected with PMA to induce arterial NET formation. TK1 protein levels were found to be increased both in PMA-treated mouse arteries (NETs vs. control: PPTK1 = 0.0403) (Fig. 7a and b) and serum (NETs vs. control: PPTK1 = 0.0015) (Fig. 7c and d). Meanwhile, the TK1 expression in serum of SHR and WKY were assessed. And the result showed that there are no difference between SHR and WKY (Fig. 5a and b, http://links.lww.com/HJH/C5).
DISCUSSION

In this study, we hypothesized that NET formation in artery probably may be related to the development of hypertension. On the basis of this hypothesis, NET formation was tested in the SHR model. Our data show that there was NET formation in the arteries of hypertensive rats. Moreover, in the mouse model, PMA was used to induce...
FIGURE 6 Exosomes derived from vascular smooth muscle cells with neutrophil extracellular trap treatment accelerate vascular smooth muscle cell proliferation via TK1 and promote vascular smooth muscle cell cycle progression. (a) Proliferation of VSMCs, VSMCs treated with exosomes derived from VSMCs and VSMCs treated with exosomes derived from VSMCs with NETs treatment were assessed by MTS assays, respectively. (b) The histogram shows statistical analysis of cell growth of VSMCs and VSMCs treated with exosomes derived from VSMCs with NETs treatment by OD values. (c) Cell cycle analysis of VSMCs, VSMCs treated with exosomes derived from VSMCs and VSMCs treated with exosomes derived from VSMCs with NETs treatment by PI staining. (d) The histogram shows statistical analysis of cell cycle of VSMCs, VSMCs treated with exosomes derived from VSMCs and VSMCs treated with exosomes derived from VSMCs with NETs treatment. (e) TK1 expression was analyzed in exosomes derived from VSMCs with NET treatment and exosomes derived from VSMCs by western blot analysis. Alix, an exosomal marker, was detected as a reference protein, and GAPDH was included as a control. (f) The histogram shows statistical analysis of TK1 expression in exosomes derived from VSMCs with NETs treatment and exosomes derived from VSMCs. (g) After TK1 knockdown in VSMCs, western blotting was performed to detect exosomal TK1 expression. (h) The histogram shows statistical analysis of TK1 expression in exosomes derived from VSMCs after TK1 knockdown. (i) Proliferation of VSMCs treated with exosomes derived from VSMCs and VSMCs treated with exosomes derived from VSMCs with TK1 knockdown were assessed by MTS assays, respectively. (j) The histogram shows statistical analysis of cell growth of VSMCs treated with exosomes derived from VSMCs and VSMCs treated with exosomes derived from VSMCs with TK1 knockdown by OD values (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). NETs, neutrophil extracellular traps; TK1, thymidine kinase 1; VSMCs, vascular smooth muscle cells.
the formation of NETs in the vascular wall, and the blood pressure of mice increased, indicating that NETs was related to the increase of blood pressure. To note, PMA also is a potent oxidative stress inducer, so the role of PMA on blood pressure should be considered.

Our in-vitro experiments demonstrated that NETs promote the G1/S transition and facilitate VSMC proliferation. Meanwhile, in this study, Ang II was used as positive control. That was as Ang II was able to promote the proliferation of vascular endothelial cells [34] and participated in the occurrence of hypertension [35]. And it was confirmed that Ang II was able to trigger the release of NETs, linking thromboinflammation with essential hypertension [36]. A label-free proteomics analysis provided insight into the molecular mechanism by which NETs promote VSMC proliferation. In particular, CDKN1b, a key kinase that suppresses cell cycle progression through the G1/S checkpoint, was downregulated in the NET-treated group and promoted the G1/S transition, consistent with the phenotypic alterations. The PI3K-Akt pathway was identified as a key pathway contributing to NET-induced alterations based on a gene ontology analysis. Previous studies have confirmed that cell proliferation can be positively regulated by the activation of the PI3K/Akt/CDKN1b-signaling axis [30]. Our findings suggest that NETs promote VSMC proliferation by accelerating the G1/S transition via the PI3K/Akt/CDKN1b-signaling axis. TK1 is a diagnostic biomarker for many types of cancer [37–40]. After the downregulation of CDKN1b, E2F promotes TK1 transcription. Our results show that TK1 was upregulated in proliferating VSMCs treated with NETs and the rapid proliferation of VSMCs was blocked by TK1 knockdown. Therefore, we conclude that TK1 promotes the proliferation stimulated by NETs via the PI3K/Akt/CDKN1b-signaling axis.

Exosomes are heterogeneous molecules with diameters of 30–200 nm. Exosomes reflect the phenotypic state of the parent cells from which they are generated. Exosomes can transfer proteins between cells and affect the biological behaviour of recipient cells through modulating signaling pathways in recipient cells [41]. Our finding demonstrated that the rapid proliferation of VSMCs induced by NETs can be transferred via exosomes. Therefore, the effects of NETs on VSMCs are spread to VSMCs with no NET contact. Furthermore, we found that TK1 is a key proliferation signaling molecule transferred by exosomes.

Pathological vascular remodeling involves changes in vascular cellular processes, such as vascular cell proliferation, apoptosis, and migration [42–45]. Owing to the high degree of plasticity, dysregulated VSMCs in the medial layer of the vessel wall are the most abundant cell type in the

![Thymidine kinase 1 expression was tested in the artery and serum of mice with neutrophil extracellular trap formation in arteries induced by phorbol-12-myristate-13-acetate. (a) Western blot analysis of TK1 expression in the superior mesenteric artery of mice injected with PMA to induce NETs formation in artery and control mice injected with physiological saline. (b) The histogram shows statistical analysis of TK1 expressed in the superior mesenteric artery of mice injected with PMA and control mice injected with physiological saline. (c) Western blot analysis of TK1 expression in the serum of mice injected with PMA and control mice injected with physiological saline. (d) The histogram shows statistical analysis of TK1 expressed in serum of mice with NETs induced and control mice.](image-url)
arterial vessel wall and are the predominant cells contributing to the pathogenesis of systemic hypertension [46,47]. Therefore, targeting dysfunctional VSMCs may have significant implications for the treatment of hypertension. NETosis promoted VSMC proliferation, which is related to vascular remodeling and hypertension. Additionally, NET formation was induced with PMA in mouse vessels. An increase in blood pressure increase was accompanied by NETosis in the small arteries of mice. NETs, therefore, was related to hypertension. Alternatively, we also demonstrated that hypertension contributed to NETosis in arteries. This is probably because of a mechanical damage to the vascular wall, which is conducive to the infiltration of neutrophils and NETosis. It is important to note that vascular remodeling related to hypertension is influenced by factors with a complex underlying mechanism. In this study, we only evaluated the effects of NETs on VSMCs. The effects of NETs on other vascular cells and the extracellular matrix should be explored in future studies.

Further, as the blood pressure increased, TK1 expression was increased in the serum of mice with PMA-induced NETosis. In cancer research, TK1 is a marker used to screen tumors, assess the response to therapy, and evaluate prognosis [48,49]. Our results extend the clinical application of TK1 by indicating that it is not only upregulated in tumors but also in benign diseases related to proliferation. The degree of TK1 up-regulation may differ among diseases and the quantification of TK1 changes in different diseases is an important topic for future research. There was no significant difference in the expression of TK1 in the serum of SHR and WKY, and there were significant individual differences in the expression of TK1 both in SHR group and WKY group. We analyze the reason is the small number of animals and differences in individual state of animals. In future experiment, we will expand the number of animals and conduct paired samples to detect the relationship between blood pressure and TK1 expression.

Broadly, our results indicate that the phenotypic alteration of VSMCs induced by NETs is a pathogenic factor in hypertension and is a promising target for antihypertensive therapy. On the basis of these findings, the management of hypertension should be largely focused on limiting the progression of complications by targeting precise pathophysiological mechanisms, such as inflammation and the vascular structure, rather than blood pressure control alone, which fails to markedly reduce the risk for vascular complications and mortality. Though more experiments are needed to explore, NETs probably become a target for prevention and treatment of hypertension.

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Conflicts of interest

There are no conflicts of interest.

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Hypertension is associated with NETs

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