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# Substance P enhances tissue factor release from granulocyte-macrophage colony-stimulating factor-dependent macrophages via the p22phox/β-arrestin 2/Rho A signaling pathway

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ABSTRACT

Granulocyte-macrophage colony stimulating factor (GM-CSF) induces procoagulant activity of macrophages. Tissue factor (TF) is a membrane-bound glycoprotein and substance P (SP) is a pro-inflammatory neuropeptide involved in the formation of membrane blebs. This study investigated the role of SP in TF release by GM-CSF-dependent macrophages.

SP significantly decreased TF levels in whole-cell lysates of GM-CSF-dependent macrophages. TF was detected in the culture supernatant by enzyme-linked immunosorbent assay after stimulation of macrophages by SP. Aprepitant (an SP/neurokinin 1 receptor antagonist) reduced TF release from macrophages stimulated with SP. Pretreatment of macrophages with a radical scavenger(pyrrolidinedithiocarbamate) also limited the decrease of TF in whole-cell lysates after stimulation with SP. A protein kinase C inhibitor (rottlerin) partially blocked this macrophage response to SP, while it was significantly inhibited by a ROCK inhibitor (Y-27632) or a dynamin inhibitor (dinasore). An Akt inhibitor (perifosine) also partially blocked this response. Furthermore, siRNA targeting p22phox, β-arrestin 2, or Rho A, blunted the release of TF from macrophages stimulated with SP. In other experiments, visceral adipocytes derived from cryopreserved preadipocytes were found to produce SP.

In conclusion, SP enhances the release of TF from macrophages via the p22phox/β-arrestin 2/Rho A signaling pathway.



## 1. Introduction

It has been demonstrated that human monocytes/macrophages produce significant procoagulant activity [1] and that granulocyte-macrophage colony-stimulating factor (GM-CSF) induces tissue factor (TF) production by these cells [2,3]. TF is a membrane-bound glycoprotein, but it also exists in a blood-borne form associated with microparticles. Thus, TF associated with both cells and microparticles in the blood provides a pool of procoagulant activity that may contribute to the initiation or expansion of thrombosis [4].

Microparticles have been implicated in the regulation of a variety of pathophysiologic processes related to coagulation/thrombosis, immune function in infectious diseases, and cancer. These particles are membrane fragments shed by virtually all eukaryotic cells upon activation or during apoptosis that display pro-inflammatory and pro-thrombotic activity after entering the circulation [5,6]. We recently reported that lipopolysaccharide (LPS) and human neutrophil elastase induced the expression of TF by monocytes [7]. In addition, it has been demonstrated that GM-CSF upregulates TF production [8], implying that the monocyte/macrophage lineage is closely associated with TF expression. Furthermore, LPS promotes the release of TF-positive microparticles [9], and stimulation of the THP-1 monocyte cell line by starvation or by LPS and calcium ionophore releases TF-bearing microparticles [10]. Formation of microparticles is associated with three major cellular events, which are release of exosomes from late endosomes, apoptotic cellular breakdown, and formation of membrane blebs [11]. While blebbing of the cell membrane is often associated with apoptosis, it may also be independently triggered by activation of membrane receptors [12–14], and microparticles are formed by detachment of blebs from the membrane [15].

In addition to initiation of coagulation by TF expressed on the cell surface, microparticles are a major source of TF and participate in thrombus formation [16,17]. It has been reported that the neurokinin 1 receptor (NK1R) mediates non-apoptotic membrane blebbing [18].

*Abbreviations:* ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRK2, G protein-coupled receptor kinase 2; NK1R, neurokinin 1 receptor; PDTC, pyrrolidinedithiocarbamate; PKC, protein kinase C; Rho A, ras homolog gene family, member A; ROCK, RhoA/Rho-kinase; siRNA, small interfering RNA; SP, substance P; TF, tissue factor.

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NK1R is a Gq-coupled receptor and its endogenous agonist is substance P (SP). Thus, formation of microparticles by cells of the monocyte/macrophage lineage may occur when membrane blebbing is stimulated through activation of NK1R by SP. Expression of the SP and NK1R genes has been identified in human peripheral blood monocytes and macrophages, and it has been suggested that SP is involved in autocrine regulation of the functions of these cells [19]. TF has been clearly linked to the existence of a procoagulant state in obesity. As well as being a neurotransmitter, SP also seems to have an important pathogenic role in obesity, and SP levels are elevated in obese individuals with type 2 diabetes [20] or obese children [21]. In human mesenteric preadipocytes, SP promotes expression of the NK1R, which is involved in proinflammatory signaling, and 3T3-L1 adipocytes and preadipocytes also express this receptor [22,23]. Moreover, circulating TF procoagulant activity is elevated in obesity [24]. These reports led us hypothesize that SP may be produced by adipocytes and may be associated with membrane bleb formation by various other cells. In the present study, we investigated SP production by cultured adipocytes derived from cryopreserved human visceral preadipocytes and the role of SP in TF release by GM-CSF-dependent macrophages.

## 2. Materials and methods

### 2.1. Ethics statement

Human peripheral blood samples were obtained from healthy volunteers and this study was approved by the Institutional Review Board of Kumamoto Health Science University. Written informed consent was obtained from all of the volunteers.

### 2.2. Chemicals and reagents

Substance P was purchased from Peptide Institute Inc. (Osaka, Japan). Human recombinant GM-CSF was obtained from Tocris Bioscience, Bristol, UK. Rottlerin (Merck Millipore, Bedford, MA), TAPI-1 (Merck Millipore), pyrrolidinedithiocarbamate (PDTC; BioVision, Mountain View, CA), LY 294002 (Cell Sigaling Technology, Boston, MA), Perifosine (Abcam Inc., Cambridge, UK), U0126 (Promega Corporation, Fitchburg, WI), Y-27632 (Wako, Osaka, Japan), and Dinasore (Abcam Inc.) were employed to study the signal transduction pathways involved in release of TF from macrophages. The actions of these reagents are summarized in Table 1.

### 2.3. Isolation of adherent monocytes from peripheral blood mononuclear cells

Lymphocyte medium for thawing (BBLYMPH1) was obtained from Zen-Bio, Inc. (Research Triangle Park, NC). Peripheral blood mononuclear cells (PBMCs) were isolated as described previously [25]. Briefly, heparinized blood samples were obtained from nonsmoking healthy volunteers and were diluted 1:1 with pyrogen-free saline. Further, PBMCs were isolated immediately after collection using Lymphoprep gradients (Axis-Shield PoC As, Norway). Then, cells were suspended with BBLYMPH1 and incubated for 3 h. For monocyte isolation by adherence, $1 \times 10^6$ cells per well were distributed into 12-well plates (Corning Inc. Costar, NY, USA) and allowed to adhere in a 5% $CO_2$ incubator at 37 °C for 2 h and washed 3 times with warm phosphate-buffered saline (PBS) to remove nonadherent cells. Then, monocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and $10 \times 10^3$ μg/L gentamicin at 37 °C in 5% $CO_2$ humidified air. The adherent monocytes were recovered with a cell scraper. The purity of monocytes was evaluated by fluorescent staining with CD14-phycoerythrin (PE) mouse anti-human monoclonal antibody (Life technologies, Staley Road Grand Island, NY) and Fluorescence Activated Cell Sorting (FACS) analysis. The recovery of monocytes was also evaluated by trypan blue staining and counted using a Zeiss microscope (Jena, Germany). Only isolated $CD14^+$ monocytes of >85% purity were used for each experiment. After monocytes were resuspended in RPMI-1640 medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 25 mM HEPES (Sigma-Aldrich), 100 mM/L L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich), the cells were stimulated with HNE for 6 h.

### 2.4. Induction of macrophages from adherent monocytes

Adherent monocytes were seeded at $1 \times 10^6$ cells/mL into 12-well tissue culture plates containing RPMI-1640 medium with 10% FCS and 4 mM L-glutamine, and were incubated in the presence of $10 \times 10^3$ ng/L recombinant human GM-CSF [26]. On day 3 and 6, the cells were washed and then fresh media containing GM-CSF was added. After further incubation on day 9, these cells were utilized in this study as GM-CSF-dependent macrophages (day 9).

### 2.5. Effect of SP on release of TF by GM-CSF-dependent macrophages

On day 9 of culture, GM-CSF-dependent macrophages were incubated for 6 h with or without SP (5 μM) and tissue factor (TF) protein levels in whole-cell lysates were determined by enzyme-linked immunosorbent assay (ELISA) with an anti-TF monoclonal antibody (Abcam Inc., Cambridge, UK).

### 2.6. Effects of rottlerin, TAPI-1, or PDTC on release of TF from GM-CSF-dependent macrophages stimulated with SP

After GM-CSF-dependent macrophages (day 9 of culture) were pretreated with rottlerin (10 μM), TAPI-1 (10 μM), or PDTC (10 μM), the cells were stimulated with SP (5 μM) for 6 h, and TF protein levels in whole-cell lysates or culture supernatants were determined by ELISA (Abcam).

### 2.7. Effects of LY294002, perifosine, U0126, Y-27632, or dinasore on TF release from GM-CSF-dependent macrophages stimulated with SP

GM-CSF-dependent macrophages (day 9 of culture) were pretreated with LY294002 (10 μM), perifosine (10 μM), U0126 (60 μM), Y-27632 (10 μM), or dinasore (10 μM). Then the cells were stimulated with SP (5 μM) for 6 h and TF protein levels in whole-cell lysates were determined by ELISA (Abcam).

### 2.8. Effect of small interfering (si) RNA for p22phox, Rho A, GRK2, or β-arrestin 2 on TF release by macrophages stimulated with SP

Transfection of GM-CSF-dependent macrophages with siRNA for p22phox (50 nM), Rho A (50 nM), GRK2 (50 nM), or β-arrestin 2 (50 nM) was performed on days 7 to 8 of culture according to the manufacturer's protocol for Lipofectamine™ RNAiMAX (Life

**Table 1**
Functional characteristics of chemical agents used.

| Chemical agents | Functions |
|---|---|
| Rottlerin | Protein kinase C inhibitor |
| TAPI-1 | Disintegrin and metalloproteinase inhibitor |
| PDTC | The radical scavenger |
| Perifosine | Akt inhibitor |
| U0126 | ERK1/2 inhibitor |
| Y-27632 | ROCK inhibitor |
| Dinasore | Dynamin inhibitor |
| Aprepitant | Substance P/NK-1 receptor antagonists |

Akt: protein kinase B.
ROCK: Rho-associated coiled-coil forming kinase.
NK-1: neurokinin 1.
ERK: extracellular signal-regulated kinase.