Original Article

Substance P induces monocyte chemoattractant protein-1 production in skin fibroblasts from genetically-diabetic mice via activation of NF-κB

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Abstract: Substance P (SP) is a prominent neuropeptide that functions in the active communication between the nervous system and immune response and pathological conditions such as inflammatory disorders and chronic wound healing. This functionality has been attributed to modulation of SP on production of inflammatory mediators, including chemokine and cytokines by binding to tachykinin receptors. In the present study, the effects of SP-induced production of chemokine MCP was examined and the activation of the proinflammatory transcription factor NF-κB was investigated as a molecular mechanism by which SP stimulates inflammatory processes underlying impaired wound healing. Using genetically diabetic mice skin fibroblasts, we showed that SP up-regulates mRNA and protein level of MCP-1 in a dose- and time-dependent manner via its receptor neurokinin-1. Moreover, this stimulatory effect requires activation of transcription factor, NF-κB, which contributed to MCP-1 gene transcription. Furthermore, we found that MG132, a NF-κB inhibitor, inhibits the phosphorylation and degradation of IκB and MCP-1 production by SP management in diabetic skin fibroblasts. Identification of the functionality exhibited by SP and its molecular mechanisms has important implications for modulation of impaired inflammation in chronic wounds and inflammatory disorders.

Keywords: Neuropeptide, tachykinin receptor, MCP-1, NF-κB activation, chronic wound

Introduction

Substance P (SP), an 11-amino acid neuropeptide member of the tachykinin family, is widely distributed (found) throughout the central and peripheral nervous systems and released by unmyelinated sensory nerve endings as well as immune cells [1, 2]. SP elicits biological responses in effector cells by binding to at least one of three distinct G protein-coupled receptors, the neurokinin (NK)-1, NK-2, and NK-3 receptors on the surface of endothelial cells, epithelial cells, and fibroblasts [3]. Several studies also confirm a major pro-inflammatory role for SP and the NK-1R in the pathogenesis and progression of inflammation of different etiologies, which is associated with pathophysiology of impaired wound healing, such as diabetic wounds [1, 4-10]. It has been reported that the pro-inflammatory responses of SP involve up-regulation both gene expression and protein synthesis of monocyte chemoattractant protein-1 (MCP-1), which is a putative chemokine for recruiting inflammatory cells into the site of wound during wound healing. However, the mechanisms for SP inducing the production of MCP-1 still remain unclear.

MCP-1, a potent chemotactic molecule, is one of the major and most studied members of the cysteine-cysteine (CC) chemokine (or beta) subfamily. MCP-1 mediates inflammatory response by recruiting inflammatory cells, such as monocytes, T lymphocytes, NK cells and others, into...
wounds site and activating these cells to fulfill their functions. Therefore, MCP-1 plays an important regulation role in normal inflammatory process and in many inflammatory disorders.

Diabetes-related impaired wounds remain a profound clinical problem with increasing prevalence and a high-risk for amputation. Previous studies showed that the characteristics included inflammatory response insufficiency at early stage and delayed wound healing, eventually [11, 12]. Importantly, the protein level of SP in the wound-healing impairment associated with diabetes was decreased by recent reports [13-15]. In addition, a study has found that MCP-1, the main chemokine in diabetic wounds, presented a low level in early stage, which indicating that SP and MCP-1 are critical in inflammation response during impaired wound-healing in diabetes [16, 17]. The external application of SP promotes wound healing by improving early infiltration of inflammatory cells in diabetic wounds and inflammatory response disorders [18-20]. The date suggests possible co-regulation of neuropeptide (SP) and chemokine MCP-1 in wound healing processes, particularly in abnormal inflammations and diabetic wounds.

We hypothesized that SP may modulate the production of MCP-1 on genetically diabetic mice skin fibroblasts and that increased MCP-1 might then be responsible for improving impaired wound healing in diabetes. In the present study, we sought to investigate the effect of inflammatory mediators, substance P, on MCP-1 production in skin fibroblasts from genetically diabetic mice, as well as its possible mechanisms.

Materials and methods

Animals

All procedures were subject to prior institutional approval by the Subcommittee for Research Animal Care at the Shanghai No. 3 Peoples’ Hospital. Four female genetically diabetic C57BL/KsJ Lepob mice weighting 20-30 g (referred to as db+/db+ mice) were obtained from Charles River Laboratories (Wilmington, USA). They were acclimatized to caged laboratory conditions for three days prior to research, then the back skin were harvested from these mice after they were sacrificed.
MCP-1 consisted of an initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 1 min. The following specific primer pairs (Shanghai Sangon Biotech, China) were used: β-actin, sense 5’TGA-CAGGATGCAGAAGGAGA3’ and antisense 5’GC-TGGAAGGTTGACGTGAG3’, MCP-1, sense 5’C-CCAGTCACTGCTGTAT3’ and antisense 5’T-CCTGAAACCTTCTGCTT3’. PCR products were analyzed using 1% agarose gel containing 0.05 mg/100 ml ethidium bromide. The result was analyzed using Mig gel analysis system, and expressed as the corrected ratio of absorbance to the β-actin absorbance in each group. The experiments were conducted independently three times.

Enzyme-linked immunosorbent assay (ELISA)

The protein levels of MCP-1 were examined using ELISA. Briefly, for SP-treated experiment, the primary cultured fibroblasts were added high glucose DMEM media (containing 0.5% FBS) for 2 h and treated with various concentrations of SP (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μM) and different times (2, 4, 8, 12, 24 and 48 h). No treatment was used as control. The supernatant was collected and centrifuged for 10 min at 2000 rpm. For MG132-treated experiment, the cells were cultured with DMEM containing 10% FBS in an incubator with 5% CO₂ at 37°C. The cells were treated with SP alone, SP plus MG132 with indicated concentration (0.5, 1, 1.25, 1.5 and 2 μM), and without treatment. The supernatant was collected for measuring MCP-1 concentration by ELISA assay according to ELISA kit manufacturer’s instruction. Absorbance was measured at 450 nm using an automatic microplate reader. The experiments were performed in duplicate, three times independently.

Western blot analysis

Western blot analysis was performed to determine the protein level of MCP-1 in the primary fibroblasts, or in the cells treated with SP, or the inhibitor MG132 plus SP, or NK-1R inhibitor L703606 plus SP. Briefly, the cells were lysed with RIPA buffer containing 1% NP-40, 0.5% deoxycholate and 0.1% SDS, PMSF. Total protein concentrations were determined by the BCA protein assay. Samples with 50 μg of total protein were loaded into 15% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membrane. Non-specific binding was blocked by incubating the membranes with 5% nonfat milk in PBST (0.05% Tween 20 in PBS). The membranes were then incubated overnight at 4°C with the primary antibodies (1:1,000) in buffer containing 2.5% nonfat dry milk. After three times wash with PBS for 10 min each, the membranes were incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:4,000) for 2 h at room temperature. The blots were developed for visualization using an enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). Semi-quantitative analysis was performed using an image analyzer with GAPDH as internal control. The experiment was repeated three times independently.

Immunofluorescence staining

Mice skin fibroblasts were inoculated in a six-well plate at 2×10³ cells/well and treated with SP, SP plus MG132, and without treatment. In SP plus MG132 management, the cells were pretreated with MG132 for 30 min, and then treated with SP for 60 min. The cells were washed three times with PBS and then fixed for 15 min with 4% paraformaldehyde. Triton was added into the cells for 30 min at room temperature. The cells were blocked with 6% fetal bovine serum for 30 min, and then incubated with primary antibody or PBS (negative control) overnight at 4°C. After washing, the samples were treated with Cy3 labeled goat anti rabbit secondary antibody for 2 hours in dark room. The slide was sealed with glycerol and analyzed under a fluorescence microscope.

Electrophoretic mobility shift assay (EMSA)

Mice skin fibroblasts were treated with SP alone, SP plus MG132, MG132 alone. For SP plus MG132 management, the primary cells were pretreated with MG132 for 30 min, and then treated with SP for 1 hour. The cells were collected for nuclear fraction. Complementary NF-κB consensus oligonucleotides (5’-AGT TGA GGG GAC TTT CCC AGG C-3’ and 5’-GCC TGG GAA AGT CCC CTC AAC T-3’) were synthesized by 1st BASE. The oligos were end-labeled with biotin separately using the biotin 3’-end DNA labeling kit and then annealed by heating to 95°C for 2 min followed by slow cooling to room temperature. Probes were stored at -20°C until use. EMSA was performed with the LightShift...
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chemiluminescent EMSA kit following the manufacturer’s instructions. Briefly, DNA binding reactions were set up by incubating 10 μg nuclear extract and 20 fmol of biotin-labeled probe together with 1 μg poly (dI-dC) for 20 min at room temperature in 20 μl of binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, and 1 mM DTT). Samples were fractionated on native 6% polyacrylamide gels in 0.5 Tris-borate-EDTA buffer and then transferred onto Zeta-Probe cationized nylon membranes. After being cross-linked by a UV-light cross-linker (Spectronics), the biotin-labeled DNA on the membranes was detected by chemiluminescence. Band shifts were visualized by exposure to X-ray films.

Statistics

The statistical analysis was performed using SPSS17.0. Student t test was used to evaluate difference between the study groups. Data was expressed as mean ± s. Statistical significance was set at P < 0.05.

Results

SP increases mRNA expression and protein level of MCP-1 in diabetic fibroblasts

Because SP has been proposed as a factor in the modulation of the inflammation. We examined whether this neuropeptide can modulate inflammatory MCP-1 production in skin fibroblasts from genetically diabetic C57BL/KsJ Lep°/° mice. SP significantly up-regulated mRNA expression of MCP-1 from 1 nM to 10 nM after 24 h, compared with control subject at 24 h (Figure 1A and 1B). At 10 nM treatment, SP stimulation of MCP-1 gene expression reached a peak level (P < 0.01).

In addition to MCP-1 gene expression, we also determine the effect of SP on the MCP-1 protein production. Diabetic skin fibroblasts were treated with various SP concentrations (from 1 nM to 1000 nM) for 24 h. Subsequently, MCP-1 protein levels were analyzed by Western blot or ELISA (Figure 2A-C). Chemokine MCP-1 level in the fibroblasts treated with 1 nM and 10 nM SP were significantly higher than that in untreated cells at 24 h (P < 0.05). The data was coincident with the MCP-1 mRNA expression results. Moreover, we observed the changes of MCP-1 by SP in different time points. SP stimulation of MCP-1 protein levels in the fibroblasts were much higher than that in non-treated cells at 12 h (P < 0.05), 24 h, and 48 h (P < 0.01) after treatment. Our data indicated that application of SP with 10 nM concentration, for 24 h substantially increases production of MCP-1 in both mRNA and protein levels. Based on these results, we decided to use 10 nM SP and incubation time of 24 h for all subsequent experiments.

NK-1 inhibitor abolishes SP stimulation of MCP-1 in diabetic fibroblasts

We next examined whether SP stimulates MCP-1 through its neurokinin-1 receptor (NK-1 receptor) [21, 22] in the genetically diabetic fibroblasts. Using pre-treatment of L703606 (a specific NK-1 receptor inhibitor) at 20 nmol for 10 min. Our data revealed that L703606 effi-
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ciently inhibited the protein level of MCP-1 that stimulated by SP in the pre-treated fibroblasts (Figure 3). The results demonstrated the specific NK-1 receptor inhibitors almost completely blocked SP-induced MCP-1 production, suggesting that MCP-1 augmentation was a result of direct activation by SP through its receptor NK-1 in genetically diabetic murine fibroblasts.

SP stimulates activation of NF-κB through NK-1 receptor in diabetic fibroblasts

To delineate the molecular mechanisms of SP-stimulated MCP-1 production, the diabetic fibroblasts were treated using 10 nM SP for different times (from 5 min up to 60 min), and then the activations of transcription factor (NF-κB) were analyzed. Because activation of NF-κB mainly occurs via IkappaB kinase (IKK)-mediated phosphorylation of inhibitory protein-IκB, the phosphorylation and degradation of I-κB is one of hallmarks for NF-κB activation.

The Western blot results showed that the amount of I-κB decreased notably after 15, 30, and 60 min after 10 nM SP stimulation (Figure 4A, upper row). In contrast, with time extension, the level of phospho-IκB increased significantly as early as 15 min, further up to 60 min, following SP stimulation (Figure 4A, mid row). The densities of blotting were indicated on

Figure 2. SP induces MCP-1 production. A. Skin fibroblasts from C57BL/KsJ Lep db mice were treated with SP (indicated concentrations), the cell lysates were harvested after 24 h incubation time and analyzed by western blotting for MCP-1. GAPDH was used to detect equal loading. B. Bar graphs show the average band density of MCP-1. C. Diabetic fibroblasts were treated with SP with indicated concentrations, the supernates were subjected to ELISA for detecting MCP-1. D. Diabetic fibroblasts were treated with SP as indicated times, the supernates were subjected to ELISA for detecting MCP-1. Mean ± SD, n = 3. *P < 0.05. **P < 0.01.
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Figure 4B, 4C. Our data suggested that stimulation of SP causes degradation of I-κB and increases of I-κB phosphorylation, which subsequently leads to NF-κB nuclear translocation. Additionally, a specific NK-1 receptor inhibitor, L703606, was used to pre-treat the cells before SP treatment. The cell lysates were subjected to Western Blotting with anti-I-κB or anti-phospho-IκB. As shown on Figure 5, L703606 inhibited distinctly the degradation of I-κB and phosphorylation of I-κB that stimulated by SP. It indicated that SP activates NF-κB via its receptor, NK-1.

To ensure SP can activate NF-κB in diabetic fibroblasts, we further analyzed translocation of NF-κB by determining the amount of nuclear factor (NF)-κB p65 in cytoplasm or in nuclear, respectively. As shown in Figure 4D-F, the amount of nuclear p65 raised greatly at 15, 30, and 60 min after SP treatment. However, the amount of p65 in cytoplasm declined obviously from 13 min to 60 min after treatment. Furthermore, an immunofluorescent staining result presented that RelA content indicated by red fluorescence was mainly found in nuclei (Figure 6, mid panel) after SP treatment, not in cytoplasm, compared to that in untreated cells (Figure 6, top panel). We used an inhibitor of NF-κB activation, MG132, to pre-treated the cells. RelA staining was mostly seen in cytoplasm after SP treatment (Figure 6, bottom panel), suggesting MG132 significantly inhibited NF-κB activation that induced by SP. These results revealed that SP treatment leads activation of NF-κB in diabetic fibroblasts.

**NF-κB activation is critical for SP-induced MCP-1 in diabetic fibroblasts**

To determine whether the activation of NF-κB could be required for SP-induced MCP-1 production, MG132, a proteasome inhibitor for inhibiting NF-κB activation was used to pre-treat cells and the amount of MCP-1 was measured by ELISA. The pre-treatment of MG132 significantly decreased the production of MCP-1, which was induced by SP, in a dose-dependent manner (Figure 7A). The above data demonstrated that NF-κB activation involved in SP-induced MCP-1 production in diabetic fibroblasts. To verify the importance of NF-κB activation for regulating MCP-1 production induced by SP, EMSA was performed in cells treated with or without MG132 plus SP. The data showed that SP activates the DNA binding activity of NF-κB (Figure 7B, lane 2), however, MG132 inhibits DNA binding activity of NF-κB induced by SP (Figure 7B, lane 3). The results suggested that NF-κB activation plays a key role in regulating SP-induced MCP-1.

**Discussion**

In this study, we investigated the molecular mechanisms by which Substance P stimulated the production of inflammatory mediator MCP-1 in skin fibroblasts from genetically diabetic
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mice. We found that Substance P stimulates MCP-1 production in a dose- and time-dependent manner via its receptor neurokinin-1 (NK-1) in diabetic skin fibroblasts. Additionally, this stimulation required activation of transcription factor, NF-κB, which contributed to MCP-1 gene transcription. These results suggest SP is not only a potent neuropeptide involved in neurogenic response, but is also a peptide activated transcription factor and modulated inflammatory mediators. To our knowledge, this is the first demonstration of SP-induced NF-κB activation leading to chemokine gene transcription activation in diabetic skin fibroblasts. Our findings contribute to the understanding of the molecular mechanisms underlying chronic in-
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Figure 5. SP induces activation of NF-κB via NK-1. A. Skin fibroblasts from C57BL/KsJ Lep\textsuperscript{db} mice were pre-treated with 20 nmol L703606 for 10 min, and then were treated by 10 nM SP for 5, 15, and 30 min. The cell lysates were subjected to western blotting for determining I-κB or phospho-IκB. GAPDH was used to verify equal loading. B, C. Bar graphs show the average band density of I-κB or of phospho-IκB. Mean ± SD, n = 3.

Figure 6. SP stimulates nuclear translocation of NF-κB. Diabetic skin fibroblasts from C57BL/KsJ Lep\textsuperscript{db} mice were untreated or treated by SP. RelA nuclear translocation was determined in the cells using immunostaining. The nuclei were stained with DAPI. The RelA is immuno-labeled in red. The top panel shows immunolabeling of untreated fibroblasts. The mid panel and bottom panel show immunostaining of 10 nM SP treated cells. The results represent one of three independent experiments. Scale bar equals 10 μm.
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Diabetic wound is known as an impaired healing model with inadequate inflammatory response in the early stage, which is required to achieve wound healing. Several studies reported that the diabetic wound impairment is associated with insufficient inflammatory cells activation and cytokine and/or growth factors secretion in the early stage [23-25]. Similar to the results, in our previous study, we observed a decreased cutaneous inflammatory cells infiltration, a lower level of inflammatory mediators, such as cytokines or chemokines, and delayed wound healing in a diabetic animal with deep partial-thickness burn injury model. Moreover, the external applications of SP improve impaired healing by enhancing inflammatory cells infiltration and inflammatory mediators secretion in diabetic injury or inflammatory response disorders [26, 27]. These results suggest that SP plays an important role in chronic and impaired wounds. In the present study, we utilized cultivated skin fibroblasts from genetically diabetic mice as a clinically relevant model that can serve to understand the molecular mechanisms underlying diabetic wound healing impairment. Our data reveal that SP up-regulates the gene expression of MCP-1 and increases MCP-1 protein level. MCP-1, as a chemokine, can recruit adequate inflammatory cells infiltration by chemoattracting neutrophils, macrophages, and T cells to wound (ulcer) site, which result in the improvement of impaired wound healing.

SP binding to its high-affinity neurokinin-1 (one of three tachykinin receptors) mediates a variety of inflammatory processes, including up-regulation of lung inflammation, microvascular leakage, and mucosal permeability [17, 19]. To examine whether SP-induced MCP-1 production is a direct effect via binding to NK-1, we used a selective nonpeptide NK1 tachykinin receptor antagonist, L703606, to block NK-1. As shown in this study, inhibition of NK-1 receptor by L703606 resulted in abrogation of MCP-1 production in diabetic skin fibroblasts. Our data suggest that interaction of SP and NK-1R mediate the process necessary for SP-induced MCP-1 production, which may therefore accelerate diabetic wound healing.

Given the function of transcription factor NF-κB in the regulation gene expression of many inflammatory mediators, it is not surprising that this transcription factor can be responsible for SP-induced MCP-1 production. SP has been reported to activate NF-κB and stimulate production of cytokines in a variety of cell types, including mast cells, macrophages, and epithelial cells [28-30]. Three mechanisms of NF-κB activation have been described, the classic pathway dependent on NF-κB inhibitory protein IκB degradation and two atypical pathways, one through the processing of p100 and...
release of p52/RelB into the nucleus and the other through the phosphorylation of p65 at multiple serine sites by protein kinases [31, 32]. Our results have identified that activation of NF-κB is involved in the SP-induced MCP-1 production. SP activated the classic NF-κB pathway as evidenced by the phosphorylation and subsequent degradation of I-κB as well as nuclear translocation of phosphorylated NF-κB. Moreover, the transcriptional dependence of MCP-1 on NF-κB by SP was confirmed with an inhibitor of NF-κB activation, MG132. Because MG132 selectively prevents I-κB phosphorylation and disrupts NF-κB function by sparing I-κB from proteasomal degradation, thereby inactivate NF-κB. With the present study, we have demonstrated that the treatment of MG132 effectively blocked NF-κB activation and significantly inhibited MCP-1 production, which stimulated by SP in diabetic skin fibroblasts. Further studies will be done to elucidate the intracellular signaling pathways between down-stream of NK-1 and upstream of NF-κB by SP stimulation.

Conclusion

In conclusion, this study suggests substance P plays an important role in impaired wound healing, such as diabetic wounds, by up-regulating chemokine MCP-1 production, via activation of NF-κB. Therefore, our findings may be beneficial for developing new strategies to treat chronic wounds and impaired healing disorders.

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Disclosure of conflict of interest

None.

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