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博士学位論文

培養ラット脊髄神経細胞における高濃度ブドウ糖による ニューロキニン 1 受容体の発現亢進

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【要　　旨】

知覚過敏は糖尿病発症早期に認められる神経障害の一つである。サブスタンス P による脊髄神経細胞におけるニューロキニン 1 受容体（NK1 受容体）の活性化が知覚過敏の原因となっている。これまでにブドウ糖濃度と NK1 受容体発現の関係についての報告はない。我々はラット脊髄神経細胞においてブドウ糖が直接 NK1 受容体発現を引き起こすという仮説を立てた。胎児ラットの脊髄神経細胞を用いてブドウ糖による NK1 受容体発現制御について検討した。高濃度ブドウ糖下においてラット脊髄神経細胞の NK1 受容体発現増加が認められた。プロテインキナーゼ A 阻害薬、プロテインキナーゼ C 阻害薬、アルドース還元酵素阻害薬は NK1 受容体発現に影響を与えなかったが、αリポ酸は高濃度ブドウ糖による NK1 受容体発現を抑制した。ミトコンドリア由来活性酸素の阻害薬は高濃度ブドウ糖による NK1 受容体発現を抑制した。これらの結果から酸化ストレスが NK1 受容体発現を制御している可能性が考えられた。糖尿病性神経障害による知覚過敏のメカニズムを説明する上で NK1 受容体発現の関与が示唆された。

キーワード　糖尿病性神経障害、知覚過敏、ニューロキニン 1 受容体、酸化ストレス

Introduction

Hyperalgesia is one of the symptoms of diabetic neuropathy, particularly in the early stages of diabetic neuropathy[1]. Substance P (SP) binds to the neurokinin1 (NK1) receptor with high affinity. Therefore, NK1 receptors are believed to be the primary target of SP that is released from neurons during neural transmission. SP activation of NK1 receptors in the spinal cord results in thermal and mechanical hyperalgesia[2]. The increase in SP binding is paralleled by an increase in NK1 receptor immunoreactivity in spinal neurons in the dorsal horn[3] and an increase in the mRNA content of the NK1 receptor in the dorsal spinal cord[4,5]. Systemic administration of a selective NK1 receptor antagonist attenuates the diabetes-induced mechanical hypersensitivity, which is mediated by central NK1 receptors in the rat[6]; this suggests that the central NK1 receptor may play an important role in diabetes-induced hyperalgesia.

The promoter region of the gene encoding the NK1 receptor contains a cyclic AMP (cAMP)-responsive element (CREB)[7]. Phosphorylation of CREB results in NK1 receptor gene expression. cAMP regulates NK1 receptor expression by phosphorylation of CREB through the activation of protein kinase A (PKA)[8]. Calcitonin gene-related peptide (CGRP) regulates NK1 receptor expression

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by elevation of intracellular cAMP, resulting in CREB phosphorylation[9]. The appearance of phosphorylated CREB in the nuclei of dorsal horn neurons blocked the N-methyl-D-aspartate (NMDA) receptor[10]. The activation of NMDA receptors by excitatory amino acids results in elevation of intracellular Ca^{2+}. CREB can also be phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase [11]. Thus, second messenger pathways that increase intracellular Ca^{2+} levels as well as cAMP may contribute to increased NK1 receptor expression in the spinal cord through an increase in the mRNA levels of the NK1 receptor.

In the diabetic state, unchecked superoxide accumulation and its resultant increases are induced as follows: increased polyol pathway activity leads to sorbitol and fructose accumulation, NAD(P)H-redox reaction is imbalanced, and changes occur in signal transduction, nonenzymatic glycation of proteins yields advanced glycation of end products, activation of protein kinase C (PKC) inhibits a cascade of stress responses, increased hexosamine pathway flux, and hyperglycemia-mediated superoxide overproduction by mitochondrial electron transport chain [12–14]. Oxidative stress is the result of an imbalance between reactive oxygen species (ROS) and antioxidants such as superoxide dismutase, catalase, glutathione, vitamin C, and vitamin E[15, 16]. Therefore, increased oxidative stress, which contributes to the pathogenesis of diabetic complications, is the consequence of either enhanced ROS production or attenuated ROS-scavenging capacity that results in tissue damage[14]. Oxidative stress induces gene expression, cell proliferation, cell apoptosis, lipid peroxidation, reduction of Na-K-ATPase, and mitochondrial dysfunction [13].

Hyperalgesia induced by diabetes in rats is attenuated by the administration of selective NK1 receptor antagonists[6], and other studies have shown that NK1 receptor antagonists can also prevent the development of inflammation-induced thermal and mechanical hyperalgesia[17,18]. However, the relationship between glucose and NK1 receptor expression has not been reported. We investigated the hypothesis that in the diabetic state, high glucose directly induces NK1 receptor expression in the rat spinal cord cells. Furthermore, we examined the reasons for NK1 receptor overexpression, taking into account diabetes-induced hyperglycemia and hyperglycemia-induced dysfunction of cellular metabolism.

Methods

Cell culture

Primary cultures of neonatal rat spinal cords were prepared as described previously[19]. Spinal cord segments from 1- to 2-day-old neonatal Wistar rats were dissected, fragmented, and dissociated by treatment with 0.25% trypsin in a calcium- and magnesium-free balanced salt solution for 15 min at 37°C. Trypsination was terminated by the addition of fetal bovine serum (FBS). The dissociated cells were centrifuged at 100 ×g for 10 min and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) by repetitive trituration. The cells were centrifuged at 100 ×g for 10 min again and resuspended in the culture medium (DMEM with 20% FBS and antibiotics; 20 ml for approximately 16 spinal cords). The cells were counted, and the cell suspension was diluted to a final concentration of 1.5×10^6 cells/ml.

Ten milliliters of the cell suspension was pipetted into 100-mm diameter Falcon Petri dishes and incubated at 37°C in a 5% CO_2 humidified atmosphere for the time required for maximum attachment of non-neuronal cells. The most enriched neuronal preparation was obtained after 3 h of preplating.

The enriched neuronal cell suspension was centrifuged at 100 ×g for 10 min. Cells were resuspended in the culture medium (DMEM with 20% FBS and antibiotics; 20 ml for approximately 16 spinal cords), counted, and plated at a density of 10^6 cells per 35-mm poly-L-lysine-coated dish. After 2 h, the cultures were transferred to serum-free medium containing N2 supplement (10 µl/ml, GIBCO), fibronectin (10 µl/ml, GIBCO), bFGF (5 ng/ml, Invitrogen), and antibiotics. The cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 8 days.

Immunohistochemistry

After culturing for 8 days, the cells were fixed by immersion in 4% paraformaldehyde in 0.15 M phosphate buffered saline (PBS) (pH 7.3, 4°C) for 20 min. The dishes

were then rinsed 3 times with 0.15 M PBS (pH 7.3) for 5 min each. Normal goat serum (5%) in 0.15 M PBS along with 1% bovine serum albumin (BSA) and 0.5% Tween 20 (pH 7.3, washing buffer) was spotted on the dishes, and these were placed in a humidified box for 30 min at room temperature. The specimens were then incubated overnight at 4°C with a primary antibody containing polyclonal rabbit anti-protein gene product (PGP) 9.5 (1:1000 dilution, N.B. Ultraclone) diluted in 0.15 M PBS with 0.1% BSA and 0.1% Tween 20 (pH 7.3, incubation buffer). The specimens were rinsed 3 times in the washing buffer, followed by incubation with fluorescein-conjugated affinity-purified goat antibody to rabbit IgG diluted at 1:500 (ICN Pharmaceuticals) in the incubation buffer and treatment with the avidin-biotin complex for 30 min at room temperature. The cells were observed by optical and fluorescence microscopy.

RT-PCR analysis

PCR primers were designed as follows: NK1 receptor (665 bp) sense 5′-CTG CTG GAT AAA CTT CTT CAG GTA-3′, antisense 5′-AGG ACA GTG ACG AAC TAT TTT CTG-3′; CGRP (318 bp) sense 5′-AAG TTC TCC CCT TTC CTG GT-3′, antisense 5′-GGT GGG CAC AAA GTT GTC CT-3′; GAPDH (532 bp) sense 5′-GGG TGG TGC CAA AAG GGT C-3′, antisense 5′-GGA GTT GCT GTT GAA GTC ACA-3′. Total RNA was extracted from the dishes using the RNeasy Mini Kit (QIAGEN). To prevent DNA contamination, the DNA was digested on a column using the RNase-Free DNase (QIAGEN). The RNA concentration was determined by measuring the absorbance at 260 nm (A260) in an ND-1000 (Nano Drop). One microgram of RNA was diluted in 20 µl of nuclease-free water. The mRNA was reverse transcribed to cDNA by using the TaKaRa RT-PCR Kit according to the manufacturer's instructions.

The resulting cDNA was amplified by PCR using the NK1 receptor primer pairs listed above. The solution was gently mixed, briefly centrifuged, and amplified by a thermal cycler. The PCR conditions for amplifying the NK1 receptor were as follows: 94°C for 5 min and 32 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The reaction products (10 µl) were then analyzed by electrophoresis in a 1.5% agarose gel containing 0.05 µl/ml ethidium bromide. The cDNAs for CGRP and GAPDH were amplified by PCR in an identical manner. The PCR conditions were as follows: CGRP 94°C for 5 min and 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s; GAPDH 94°C for 5 min and 22 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min.

Western blot analysis

Cells that had been cultured for 8 days were mixed with 1% sodium dodecylsulfate (SDS) sample loading buffer. Protein concentrations of the samples were measured and calculated using the BCA Protein Assay Kit (Pierce). Estimations were made based on the absorbance at 562 nm. The final concentration of the sample protein was adjusted to 1 mg/ml. Aliquots of the crude lysate containing 40 µg of protein were mixed with 2× SDS sample buffer, heated to 95°C for 10 min, and then electrophoresed on a 10% Bis Tris gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk in PBS-Tween 20 (PBST; 0.05% Tween 20 in PBS) for 1 h, the membrane was incubated overnight with anti-NK1 receptor (anti-NK-1R, rabbit polyclonal antibody, 1:2000, Santa Cruz Biotechnology) or anti-β-actin (Monoclonal Anti-β-Actin Clone AC-74, SIGMA). It was then washed 3 times with PBS for 15 min each and incubated for 1 h at room temperature with peroxidase-labeled anti-rabbit antibody (1:2000, Amersham Biosciences) or ECL peroxidase-labeled anti-mouse antibody (1:2000, Amersham Biosciences) in 5% non-fat dry milk. The membranes were washed 3 times with PBS for 15 min each. Antigen-antibody complexes were visualized using ECL Western Blotting Detection Reagents (Amersham Biosciences); the membranes were then analyzed using a luminescent image analyzer (LAS-3000, FUJIFILM).

Measurement of cAMP

Cells were incubated for 8 days. The medium was aspirated from the plate, and the cells were treated with 1 ml of 0.1 M HCl, following which they were incubated for 20 min. The cells were scraped with a cell scraper,

and the mixture was dissociated by pipetting up and down. Subsequently, they were centrifuged at 1000 ×g for 10 min. The supernatant was decanted into a clean test tube. The cAMP assay was performed using a sensitive protein binding EIA kit (Cayman Chemicals).

Dihydroethidium staining

The oxidative fluorescent dye dihydroethidium was used to evaluate the intracellular production of superoxide. Dihydroethidium is a cell-permeable dye that reacts with superoxide ions to form ethidium; this in turn intercalates in the DNA and provides nuclear fluorescence at an excitation wavelength of 520 nm and an emission wavelength of 610 nm. The cells were incubated at 37°C in phenol red-free Hanks medium containing dihydroethidium (10 µmol/l). After 15 min, the cells were rinsed in phenol red-free Hanks medium, and images were obtained by fluorescence microscopy. Fluorescence was detected with a 585-nm long-pass filter.

Data analysis

Data were expressed as mean±SE of multiple experiments. Student's t-test was used to compare between the 2 groups, and ANOVA (Scheffe's F test) was used to compare among more than 3 groups for multiple comparisons. P values < 0.05 were considered statistically significant.

Results

Isolated cells from the spinal cord of neonatal rats were incubated for 8 days; the cells were then stained with PGP 9.5, an antigen specific for neuronal cells. The cells were observed using both optical and fluorescent microscopes (Figures 1a, 1b). To examine the proportion of neuronal cells, we counted all cells and PGP 9.5-immunopositive cells in the same 20 fields. Approximately 85% of total cells were stained with PGP 9.5, and these neuronal cells were used for the following experiments.

NK1 receptor expression was significantly increased in cells cultured in high glucose than in low glucose. We evaluated whether high glucose could induce NK1 receptor expression in cultured spinal cord cells. Cells were treated with either low glucose (5.6 mmol/l) or high glucose (25 mmol/l) for 8 days. The RNA extracted from these cells was subjected to relative RT-PCR analyses using GAPDH as the internal control. The results show that high glucose treatment (1.235 ± 0.209, n = 18) increased NK1 receptor expression to a greater extent than low glucose treatment (0.821 ± 0.220, n = 18) (Figures 2a, 2b). NK1 receptor expression increased in a time-dependent manner (data not shown). A few NK1 receptors were observed in cells at 2 h after the initial incubation, and their numbers gradually increased in a time-dependent manner. We prepared culture

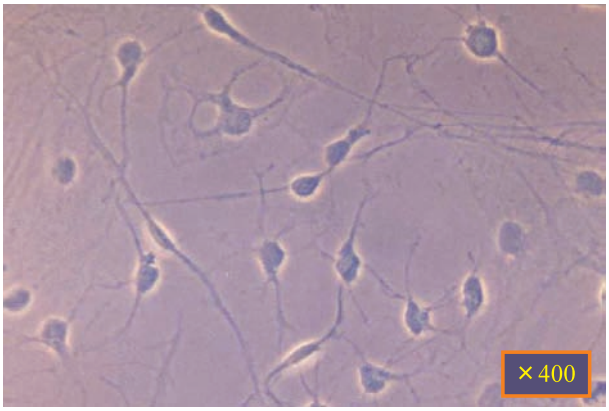

Figure 1a

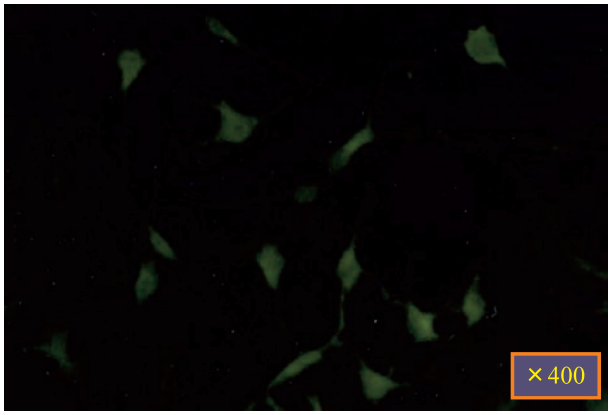

Figure 1b

Figure 1　mmunohistochemistry using PGP 9.5, an antigen specific for neuronal cells. Figure 1a shows the image obtained by an optical microscope, and Figure 1b shows the image obtained by a fluorescent microscope. Neuronal cells stained with PGP 9.5 are visualized in green. Approximately, 85% of total cells were stained with PGP 9.5. Photographs are at 400× magnification.

media containing 5.6 mmol/l, 15.2 mmol/l, 25 mmol/l, and 34.7 mmol/l D-glucose. A significant increase in NK1 receptor expression was observed in cells cultured in 25 and 34.7 mmol/l glucose (Figure 2c).

The increase in NK1 receptor expression due to high glucose was not due to osmotic pressure. The possibility that osmotic pressure increased NK1 receptor expression was excluded by using L-glucose (which is not metabolized in cells but has osmotic activity). We prepared culture media containing 5.6 mmol/l D-glucose, 25 mmol/l D-glucose, and 5.6 mmol/l D-glucose + 19.4 mmol/l L-glucose. The cells were cultured in each medium for 8 days. The group cultured in 25 mmol/l D-glucose or in 5.6 mmol/l D-glucose + 19.4 mmol/l L-glucose had the same osmotic pressure. NK1 receptor expression was significantly increased in the group cultured in 25 mmol/l D-glucose (0.610 ± 0.133, n = 6), but not in 5.6 mmol/l D-glucose + 19.4 mmol/l L-glucose (0.385 ± 0.162, n = 6) (Figure 2d); this suggests that osmotic pressure does not affect NK1 receptor expression.

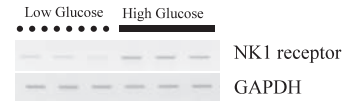

Figure 2a

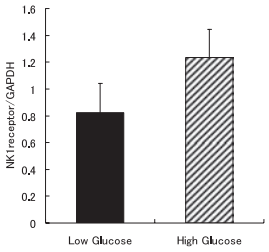

Figure 2b

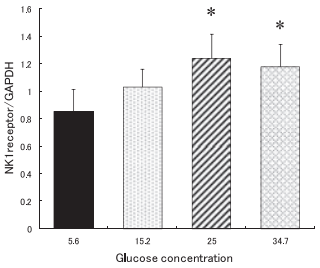

Figure 2c

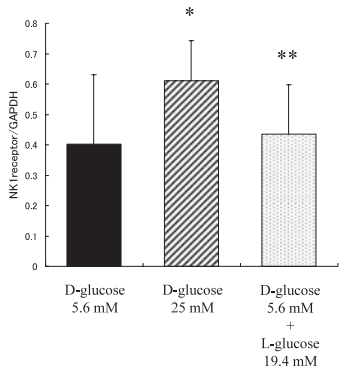

Figure 2d

Figure 2 NK1 receptor expression was significantly greater in cells cultured in high glucose than in those cultured in low glucose (a,b). Data are means ± SE. Densitometric quantification of the corresponding bands was performed using the NIH image software. *p < 0.01 vs. low glucose group (n = 18). NK1 receptor expression was increased in a concentration-dependent manner (c). *p < 0.05 vs. 5.6 mmol/l glucose concentration group (n = 6). Increase in NK1 receptor expression due to high glucose was not due to osmotic pressure (d). *p < 0.05 vs. 5.6 mmol/l glucose concentration group (n = 6). **p < 0.05 vs. 25 mmol/l glucose concentration group (n = 6).

We examined NK1 receptor expression by Western blotting. Figures 3a and 3b show that in a protein assay, NK1 receptor expression was increased in high glucose;

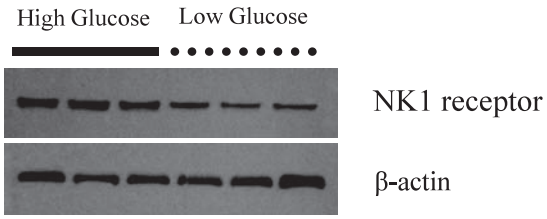

Figure 3a

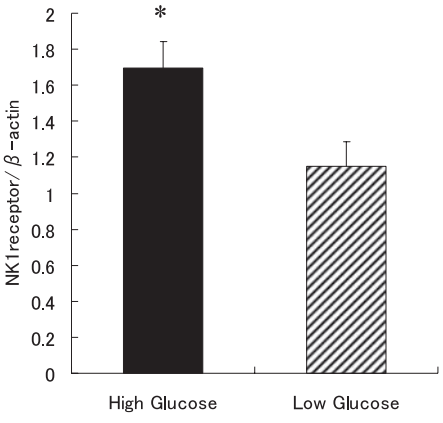

Figure 3b

Figure 3 The bands obtained by Western blot analysis of cells treated with high and low glucose concentrations are shown in (a). In a protein assay, NK1 receptor expression was significantly greater in cells cultured in high glucose than in those cultured in low glucose (b). *p < 0.05 vs. low glucose group (n = 6).

this indicates that NK1 receptor expression was increased in cells treated with high glucose.

Treatment of cultures with dibutyryl-cAMP (dbcAMP) resulted in an increase in NK1 receptor expression. Cells treated with dbcAMP for 4 h exhibited increased NK1 receptor expression in a concentration-dependent manner. Although this increase was observed in cells treated with high and low glucose, the increase in NK1 receptor expression was significantly greater in cells treated with high glucose than in those treated with low glucose at any dbcAMP concentration. In cultures with high glucose, the increase in NK1 receptor expression after treatment with 1 mM and 10 mM dbcAMP was 155% and 233% that of the nontreated control. In cultures with low glucose, this increase after treatment with 1 mM and 10 mM dbcAMP was 212% and 248% that of the nontreated control (Figure 4a). After treatment with 10 mmol/l dbcAMP, a greater increase in NK1 receptor expression was observed in the high-glucose culture than in the low-glucose culture. In our experiments, NK1 receptor expression in cell culture was further increased by the presence of high glucose after treatment with dbcAMP in a concentration-dependent manner.

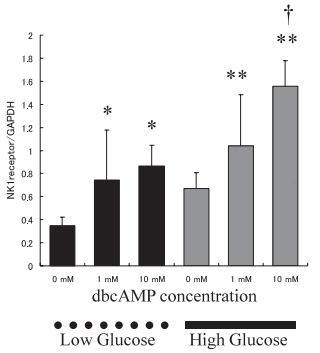

Figure 4a

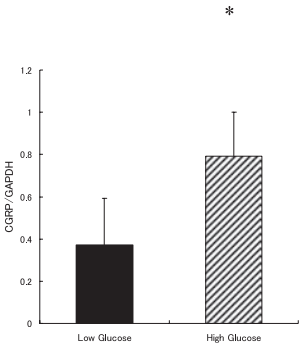

Figure 4b

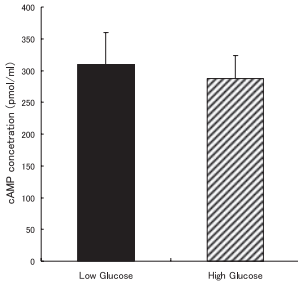

Figure 4c

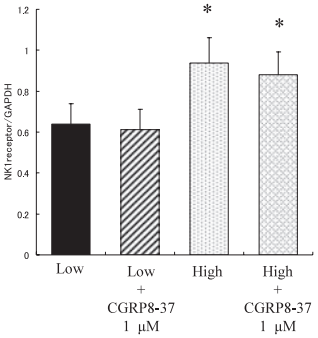

Figure 4d

Figure 4 dbcAMP induced the increase in NK1 receptor expression in cells treated with high and low glucose. This increase in NK1 receptor expression occurred in a concentration-dependent manner (a). *$p < 0.01$ vs. untreated low glucose group ($n = 6$). **$p < 0.01$ vs. untreated high glucose group ($n = 6$). †$p < 0.01$ vs. low glucose group treated with 10 mM dbcAMP ($n = 6$). CGRP expression was significantly greater in cells cultured in high glucose than in those cultured in low glucose (b). *$p < 0.01$ vs. low glucose group ($n = 12$). Intracellular cAMP in cells was not changed by the glucose concentration (c). The increase in NK1 receptor expression due to the high glucose concentration was not suppressed by treatment with CGRP8-37 (d). *$p < 0.05$ vs. untreated low glucose group ($n = 6$).

Although a greater increase in CGRP was observed in cells cultured in high glucose than in those cultured in low glucose, cAMP was not increased in the cultured cells. We measured CGRP gene expression using RT-PCR, and it had increased in cells cultured in high glucose (Figure 4b). Since an increase in CGRP induces an increase in intracellular cAMP in the cell, which results in NK1 receptor expression, we measured intracellular cAMP using ELISA. The intracellular cAMP amount was not increased in cells cultured in high glucose (Figure 4c). To exclude the possibility that the increase in intracellular CGRP induced NK1 receptor expression, we measured NK1 receptor expression by RT-PCR using a CGRP receptor antagonist ($CGRP_{8\text{-}37}$). The increase in NK1 receptor expression due to high glucose was not suppressed by $CGRP_{8\text{-}37}$ treatment (Figure 4d).

The increase in NK1 receptor expression due to high glucose was suppressed by antioxidant treatment. To examine the reasons for the increase in NK1 receptor expression, we prepared a culture medium containing a PKA inhibitor (protein kinase A inhibitor 14-22 amide, 10 μmol/l), a PKC inhibitor (staurosporine, 1 μmol/l), an antioxidant (α-lipoic acid (α-LA), 500 μmol/l), and

an aldose reductase inhibitor (ARI, epalrestat, 1 μmol/l). The cells were incubated for 7 days and then transferred to a medium containing each inhibitor, following which incubation was carried out for 24 h. The RNA extracted from these cells was subjected to relative RT-PCR analyses using GAPDH as the internal control. The increase in NK1 receptor expression was suppressed by α-LA (Figure 5a). This suppression of NK1 receptor expression was observed to occur in an α-LA concentration-dependent manner (Figure 5b). In a protein assay, the increase in NK1 receptor expression was suppressed by treatment with α-LA (Figure 5c). The increase in NK1 receptor expression was not suppressed by other inhibitors. This result suggests that the increase in NK1 receptor expression was suppressed by an antioxidant.

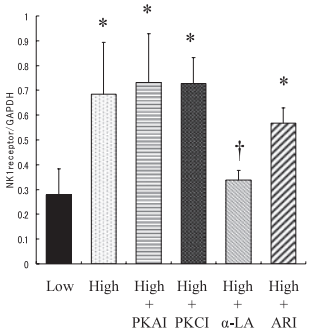

Figure 5a

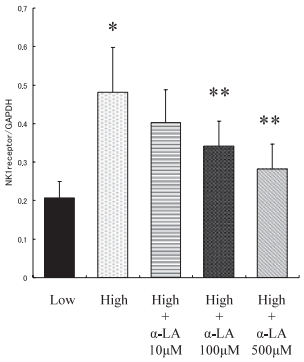

Figure 5b

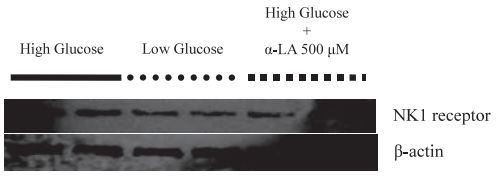

Figure 5c

Figure 5 The increase in NK1 receptor expression due to the high glucose concentration was suppressed by treatment with α-LA (a). We prepared a culture medium containing a PKA inhibitor (protein kinase A inhibitor 14-22 amide, 10 μmol/l), a PKC inhibitor (staurosporine, 1 μmol/l), an antioxidant (α-LA, 500 μmol/l), and an aldose reductase inhibitor (ARI, epalrestat, 1 μmol/l). The cells were incubated for 7 days and then transferred to a medium containing each inhibitor and were further incubated for 24 h. *p < 0.05 vs. low glucose group (n = 6). **p < 0.05 vs. untreated high glucose group (n = 6). NK1 receptor expression was suppressed in an α-LA concentration-dependent manner (b). *p < 0.05 vs. low glucose group (n = 6). †p < 0.05 vs. nontreated high glucose group (n = 6). The bands observed in Western blot analysis of cells treated with high glucose, low glucose, and high glucose with 500 μM α-LA are shown in (c).

Oxidative stress was caused by overproduction of ROS by the mitochondrial electron transport chain. To investigate the productivity of ROS in cultured cells, we prepared a culture medium containing the following inhibitors: NADPH oxidase inhibitor (apocynin, 30 μmol/l), glutamine fructose-6-phosphate amidotransferase inhibitor (azaserine, 5 μmol/l), and mitochondrial complex II inhibitor that blocks mitochondrial superoxide production (TTFA, 10 μmol/l). After incubating the cells for 7 days,

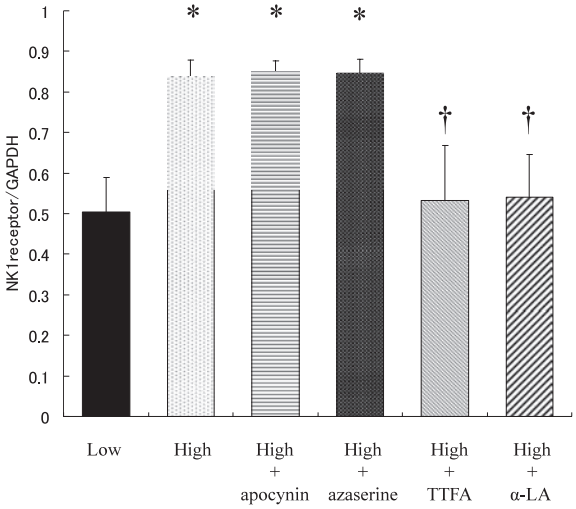

Figure 6 We prepared a culture medium containing the following inhibitors: NADPH oxidase inhibitor (apocynin, 30 μmol/l), glutamine fructose-6-phosphate amidotransferase inhibitor (azaserine, 5 μmol/l), mitochondrial complex II inhibitor that blocks mitochondrial superoxide production (TTFA, 10 μmol/l), and α-LA, 500 μmol/l. The cells were incubated for 7 days and then transferred to a medium containing each inhibitor and were further incubated for 24 h. *p < 0.01 vs. low glucose group (n = 6). †p < 0.01 vs. untreated high glucose group (n = 6).

the culture was transferred to a medium containing each inhibitor, and the cells were further incubated for 24 h. The RNA extracted from these cells was subjected to relative RT-PCR analyses using GAPDH as the internal control. The increase in NK1 receptor expression was suppressed by treatment with TTFA and α-LA (Figure 6); however, it was not suppressed by other inhibitors. This result suggests that the elevated intracellular oxidative stress was due to activation of the mitochondrial electron transport chain.

Oxidative stress was increased in cells cultured in high glucose, and this increase was suppressed by α-LA treatment. Using dihydroethidium staining, we determined the oxidative stress in cells cultured in high glucose with or without α-LA treatment. Dihydroethidium staining increased in cells cultured in high glucose, whereas those cultured in low glucose showed low staining levels. The cells cultured in high glucose with α-LA treatment (1000 μmol/l) showed levels of dihydroethidium staining that were almost similar to those observed in the low-glucose culture (Figure 7); this suggests that the oxidative stress induced by high glucose was suppressed by α-LA.

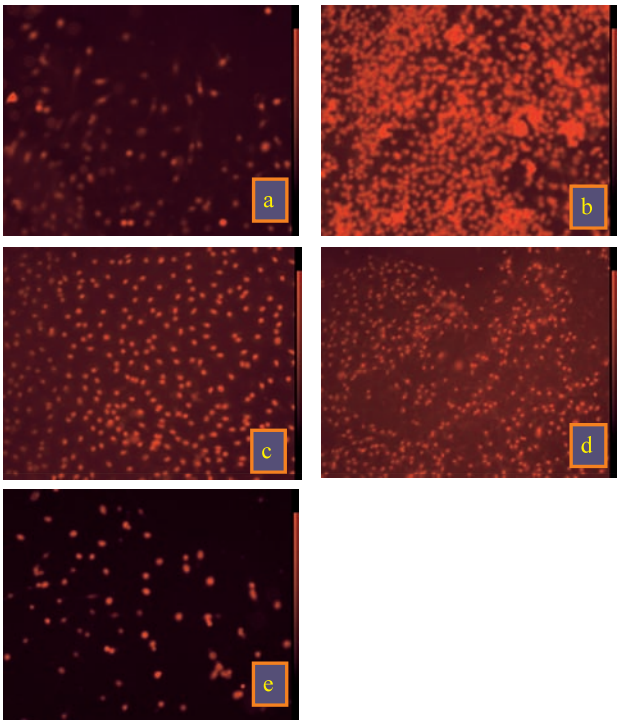

Figure 7 Detection of superoxide production by dihydroethidium staining in cultured cells. Representative results of dihydroethidium staining are shown for cells treated with low glucose (a), high glucose (b), high glucose with 10 μM α-LA (c), high glucose with 100 μM α-LA (d), and high glucose with 1000 μM α-LA (e). Photographs are at 100× magnification.

Discussion

These studies provide evidence that glucose regulates NK1 receptor expression in neonatal rat spinal cord. The increase in NK1 receptor expression is not due to osmotic pressure. Since this increase is suppressed by antioxidants, the increase in NK1 receptor expression may be caused by an increase in intracellular oxidative stress. In our study, the increase in intracellular ROS induced by glucose was derived from the activation of the mitochondrial electron transport chain. We showed that cells cultured under high glucose condition undergo excessive oxidative stress, and this increase in oxidative stress is suppressed by antioxidants. Considering these results, we speculate that high glucose concentration increases NK1 receptor expression through an excessive intracellular oxidative stress. Although our studies do not exclude the possibility that high glucose or oxidative stress inhibits the degradation of mRNA, we showed that in a protein assay, the increase in NK1 receptor expression is observed in cells cultured under high glucose condition; this suggests that NK1 receptor gene expression and the consequent production of NK1 receptor proteins certainly exist. NK1 receptor expression is regulated by cAMP[8], activation of the CGRP receptor[9], intracellular Ca^{2+} [20], activation of the NMDA receptor [21, 22], and Ca^{2+}/calmodulin-dependent protein kinase[11]. Thus, second messenger pathways that increase intracellular Ca^{2+} levels as well as cAMP may contribute to increased NK1 receptor expression in the spinal cord through an increase in the mRNA levels of the receptor protein. We showed that high glucose also induces an increase in NK1 receptor expression, which is due to oxidative stress.

The increase in NK1 receptor expression occurring under high glucose conditions is not caused by CGRP and CGRP receptor activation. In our study, CGRP production was also increased in cells treated with high glucose. CGRP and CGRP receptor activation induced NK1 receptor

expression through an increase in intracellular cAMP and PKA activation [23]. In our study, intracellular cAMP was not increased in cells treated with high glucose, and the increase in NK1 receptor expression was not suppressed using a PKA inhibitor. Furthermore, the increase in NK1 receptor expression was not suppressed using a CGRP receptor antagonist. These results suggest that the increase in NK1 receptor expression due to high glucose is not related to CGRP receptor activation and its intracellular signaling that leads to NK1 receptor gene expression.

Oxidative stress induces an increase in NK1 receptor expression. Since this is suppressed by antioxidant treatment, oxidative stress may have the ability to induce NK1 receptor gene expression. Although this mechanism was not clarified in our study, oxidative stress has been implicated in the pathogenesis of diabetic complications [16, 24, 25]. There is a growing body of literature that links cellular oxidative stress to the activation of mitogen-activated protein (MAP) kinases [26–32]. The activation of MAP kinases may result in CREB phosphorylation in the NK1 receptor. Another possibility is that oxidative stress induces the increase in intracellular Ca^{2+}; this results in phosphorylation of CREB in the NK1 receptor through the activation of Ca^{2+}/calmodulin-dependent protein kinase. Since intracellular Ca^{2+} was not measured in our study, we cannot deny the relationship between oxidative stress and intracellular Ca^{2+} concentration. However, it is certain that excessive oxidative stress has the ability to induce NK1 receptor expression.

Spare NK1 receptors may play an important role in the spinal cord in maintaining responsiveness to SP during peripheral inflammation when persistent release of SP contributes to increased excitability of spinal neurons [33–36]. The spare receptors may be particularly important based on the premise that the recycling of the NK1 receptor through intracellular pathways occurs in response to ligand activation [37]. Selective antagonists of the NK1 receptor attenuate the mechanical hypersensitivity state in diabetic rats [6]. In diabetic patients, hyperglycemia may induce NK1 receptor expression in neuronal cells in the spinal cord, resulting in hyperalgesia. Our study indicates that antioxidants such as α-LA may constitute an alternative therapy for diabetes-induced hyperalgesia.

Conclusion

NK1 receptor expression was increased in rat spinal neuronal cells cultured in high glucose. The expression of NK1 receptor was suppressed by antioxidants. This indicates that oxidative stress is involved in the expression of NK1 receptor.

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High Glucose Increases the Expression of Neurokinin1 Receptors in Cultured Neonatal Rat Spinal Neurons

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Summary

Hyperalgesia is one of the symptoms of diabetic neuropathy, particularly in the early stages of diabetic neuropathy. The activation of neurokinin1 (NK1) receptors in the spinal cord by substance P results in thermal and mechanical hyperalgesia. The relationship between glucose and NK1 receptor expression has not been reported. We investigated the hypothesis that in the diabetic state, high glucose directly induce NK1 receptor expression in rat spinal neuronal cells. We used primary cultures of neonatal rat spinal neurons to elucidate whether NK1 receptor expression is regulated by glucose. NK1 receptor expression increased in cells cultured under a high glucose condition. Although inhibitors of protein kinase A, protein kinase C, and aldose reductase did not affect NK1 receptor expression, α-lipoic acid suppressed it under high glucose conditions. A specific inhibitor of mitochondrial complex II also suppressed the increase in NK1 receptor expression. These results indicate that high glucose increases NK1 receptor expression, which is due to oxidative stress and NK1 receptor expression contributes to mechanisms underlying hyperalgesia in diabetic neuropathy.

Key words diabetic neuropathy, hyperalgesia, neurokinin1 receptors, oxidative stress

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