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Effect of Sigma-1 Receptors on Voltage-Gated Sodium Ion Channels in Colon Cancer Cell Line SW620

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Abstract

Background: Voltage-gated sodium channels (VGSCs) play pivotal roles in the metastatic process in several cancers, including breast and colon cancers. Sigma-1 receptors are known to interact and form complexes with a number of ion channels aiding the delivery of the channel protein to the plasma membrane. Drugs that bind the Sigma-1 receptor are hypothesized to affect this process and reduce the delivery of the channel protein to the plasma membrane, in turn reducing the metastatic potential of the cells.
Methods: Human colon cancer cell line SW620 was utilized as a model to investigate the interaction between the neonatal VGSC (nNav1.5) and the Sigma-1 receptor. This was accomplished using drugs that bind the Sigma-1 receptor, Sigma-1 receptor silencing, and antibodies that bind and block the nNav1.5 channel.
Results: Sigma-1 receptor drugs SKF10047 and dimethyl tryptamine were found to alter (reduce) the adhesion of these cells by 46–54% at a 20 μM drug concentration. In a similar manner, gene silencing of the Sigma-1 receptor had a similar effect in reducing the adhesion of these cells to collagen-coated plates by 30%. The Sigma-1 receptor was found to be in a complex with nNav1.5 in SW620 cells, and Sigma-1 drugs or gene silencing of the Sigma-1 receptor results in a reduction of the surface expression of nNav1.5 by $\sim$50%. Culture of SW620 cells under hypoxic conditions resulted in upregulation of the Sigma-1 receptor and nNav1.5. In addition, surface expression of nNav1.5 protein increased under hypoxic culture conditions and this was inhibited by the application of SKF10047.
Conclusions: It is proposed that in colon cancer cells, upregulated Sigma-1 receptor expression in hypoxia led to increased nNav1.5 protein expression at the plasma membrane and resulted in the cells switching to a more invasive state.

Keywords: Sigma-1 receptor, sodium channel, colon cancer

Introduction

Ion channels play many essential physiological roles in the human body. In the past 20 years, their importance in many features of cancer cell biology, including proliferation, adhesion, migration, and invasion, has been reported. Given that ion channels are also pharmacological targets, many researchers have attempted to utilize drugs that modulate ion channels to target specific processes that these ion channels have been demonstrated to act upon.[1] Within cancer cells, we are starting to elucidate the exact mechanisms of how ion channels may alter the cellular pathophysiology. Functional expression of voltage-gated sodium channels (VGSCs) in breast, prostate, lung, colon, and cervical (and other) cancers has been reported by many groups.[2–10] What has come to light is that VGSC expression appears to be a ''switch'' that results in cancer cells becoming highly invasive.[11]

VGSCs can be targeted/blocked with pharmacological agents and, utilizing mouse models, it was shown that this results in suppression of local invasion and metastasis.[12,13] For breast and colon cancer cell lines, a neonatal splice version Nav1.5 (nNav1.5) appears to be the predominant subtype of VGSC.[6,11] In the colon cancer cell line SW620, hypoxia drives invasiveness, possibly through the persistent current component of nNav1.5.[6] In a previous publication, we have documented that in breast cancer cell lines, nNav1.5 channels interact with and are modulated by Sigma-1 receptors[14] and this interaction alters the metastatic potential of these cells.

Sigma-1 receptors are transmembrane proteins that appear to act as molecular chaperones. The Sigma-1 receptor is present in multiple locations within the cell, including in the endoplasmic reticulum, plasma membrane, and perinuclear areas.[15] The chaperone activity of Sigma-1 receptors affects

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various ''client'' proteins, which include the IP3 receptor, voltage-gated ion channels, and ankyrin (for reviews[15,16]). The natural ligand for these receptors remains elusive but some evidence indicates that dimethyl tryptamine (DMT) may interact with Sigma-1 receptors in the brain.[17,18] However, a wide range of antidepressants, antipsychotics, opiates, and alkaloids also bind to Sigma-1 receptors with a variety of affinities.[19,20]

There have been many studies documenting ion channel modulation by Sigma-1 receptors. This modulation is reported to be independent of G-proteins, cytoplasmic factors, and protein phosphorylation.[21] Patch clamp studies indicate that for modulation to occur, the ion channel and Sigma-1 receptor must be in close proximity most likely in a molecular complex.[22] The VGSC found in cardiac cells (Nav1.5) is modulated by Sigma-1 receptors.[23] Furthermore, Sigma-1 receptors and Nav1.5 channels form complexes with a four-fold symmetry as revealed by atomic force microscopy.[24] Within MDA-MB-231 cells, The Sigma-1 receptor was found to form complexes with Nav1.5 in these cells by immunoprecipitation experiments and modulate the current density of the channels.[24] This led to a hypothesis according to which Sigma-1 receptor ligands act on the Sigma-1 receptor and by doing so change the invasive potential of breast cancer cells in association with Nav1.5 channels.[14]

Hypothetically, under normal physiological conditions, Sigma-1 receptors are inactive, but under cellular stress act to modulate ''client'' proteins such as voltage-gated ion channels, altering the electrical plasticity of the cells and hence promoting cellular survival.[25] Support for this hypothesis include studies that Sigma-1 silencing suppresses recovery following experimental stroke[26] and promotes retinal degeneration after acute optic nerve damage.[18] A comparable mechanism by which cancer cells utilize the Sigma-1 receptor to modulate ion channels and alter their behavior in response to the tumor microenvironment has been proposed.[16]

Given the demonstrated role of nNav1.5 in the metastasis of colon cancer cells[6] and previous experimentation in breast cancer cells indicating a functional link between nNav1.5 and Sigma-1 receptors, we aimed (1) to investigate Sigma-1 receptors and nNav1.5 in colon cancer cells and their interaction and (2) to elucidate whether the action of Sigma-1 drugs on colon cancer cells could occur via an interaction with nNav1.5 and hence alter the invasive properties of these cells.

Methods

Cell culture

The human colon cancer cell line SW620, which was obtained from LGC Standards (Middlesex, UK), was maintained in Dulbecco's modified Eagle's medium (DMEM) with the addition of 5% fetal calf serum, 4 mmol/L glutamine, and antibiotics (penicillin/streptomycin). This cell line was cultured in a 37°C carbon dioxide (CO_2) incubator and split every 7 days. Hypoxia (1% oxygen) was induced for 24 h in a dedicated incubator (Micro Galaxy; RS Biotech Laboratory Equipment Ltd., Irvine, UK).

Pharmacological agents

Tetrodotoxin (TTX) was obtained from Tocris (Bristol, UK). Sigma-1 receptor drugs SKF10047 and 5-methyl-DMT were suspended with a 50% mixture of ethanol and water. Control cells were also treated with this mixture. Both Sigma-1 receptor ligands were obtained from Sigma-Aldrich (Gillingham, UK).

Antibodies

A polyclonal antibody to nNav1.5 was produced as described previously.[14] The Sigma-1 receptor antibody was described previously.[27] Hif-1 antibody (H1alpha67 monoclonal) was obtained from Thermo Fisher (Hemel Hempstead, UK). The mouse anti-Nav1.5 antibody was obtained from Abcam (Cambridge, UK).

Gene silencing

Construction and verification of vectors to silence Sigma-1 receptors utilizing the pSilencer RNAi vector were described previously.[27] A Sigma-1 randomized sequence in the same vector was utilized as a control. FuGENE HD (Roche, Welwyn Garden City, UK) and Opti-MEM (Thermo Fisher) were used to transfect the vectors into SW620 cells and efficiency was monitored using the plasmid pHr-GFP, which was co-transfected into cells. Silencing was verified by Western blotting and the efficiency was quantified using a cell-based enzyme-linked immunosorbent assay (ELISA) (see details in Cell ELISA protocol section) with an anti-Sigma-1-specific antibody. Overall silencing efficiencies of more than 80% were achieved.

Proliferation assay

Cells at a concentration of 0.5×10^5 cells per mL in maintenance media were aliquoted into 24-well plates. After 16 h, the media were replaced with 1 mL of media containing SKF10047, N-methyl-DMT, or control treatment. After 72 h at 37°C, cell proliferation was determined using a crystal violet assay. Cell proliferation was quantified by measuring absorbance at 50 nm using growth media only as a blank.

Crystal violet assay

Medium was aspirated and the cells were washed three times with phosphate-buffered saline (PBS). Crystal violet stain (5 mg/mL in 20% methanol) was added to each well (500 μL/well) and incubated for 10 min at room temperature. Subsequently, the cells were washed three times by carefully submerging them in a tank containing water. The plates were inverted and tapped dry on tissue paper. Subsequently, a solubilization buffer (1% sodium dodecyl sulfate [SDS]) was added (500 μL/well) and the plates were shaken for 30 min at room temperature. The absorbance of each plate was measured using a plate reader set at 570 nm. As a control, blank wells without cells were also stained.

Adhesion assay

Cells were plated into 100 mm plates at a concentration of 1.5×10^6 cells in 10 mL of maintenance media and incubated for 16 h. Following this media with Sigma-1 drugs or control treatment or in some experiments, gene silencing was performed as described above. After 24 h at 37°C, cells were washed with PBS and subsequently treated with PBS, which contained 10 mM ethylenediaminetetraacetic acid (EDTA)

for 15 min at 37°C. Following detachment, the cells were centrifuged at 1200 rpm for 5 min. The cell pellet was suspended in 2 mL of growth media and quantified. The cells were counted and adjusted to 5×10^5 cells per mL. Twenty-four-well plates, which had been precoated with collagen, were seeded with the cell suspension at a concentration of 2.5×10^5 cells per well and incubated for 60 min at 37°C. The wells were washed three times with PBS and inverted onto filter paper. Crystal violet was used to stain the cells and absorbance at 570 nm was determined. Collagen coating of plates occurred using 125 μg/mL of collagen in PBS for 1 h at 37°C. Subsequently, the plates were washed three times with PBS followed by once with DMEM.

Transverse migration assay

PBS with 10 mM EDTA was used to detach the SW620 cells following incubation in media without serum for 24 h. The cells were resuspended following centrifugation in media free of serum at a concentration of 2×10^6 cells per mL. To the top chamber of 8-μm pore, 24-well migration plates (Sigma-Aldrich), 100 μL of cells, were pipetted with either SKF10047 or DMT, and to the bottom chamber, 500 μL of maintenance media was added. Control wells without drugs were also included. Cells were incubated at 37°C for 24 h. Following incubation, media were removed from the top chamber and washed with PBS. Subsequently, the bottom chamber was also aspirated and washed with PBS, which was replaced with 500 μL of PBS-EDTA containing 10 μM calcein-AM (Thermo Fisher) and incubated for 1 h at 37 C in a CO_2 incubator. Plates were shaken for 10 min at room temperature and following this the top chamber was removed and the plate was read at 480 nm excitation and 520 nm emission in a fluorescence plate reader.

Western blotting

Protein extracts from SW620 cells were prepared utilizing an RIPA buffer containing 1% Triton-X and a mix of proteinase inhibitors (Roche). Extracts from cells were adjusted to a concentration of 1 mg/mL, mixed with SDS sample buffer (Thermo Fisher), incubated at 95°C for 10 min, and 10 μg of total protein was pipetted onto Tris-glycine acrylamide gradient minigels (Thermo Fisher). Following separation for $\sim$1 h, the proteins in the gels were transferred to polyvinylidene difluoride membranes for 2 h (for Sigma-1 receptor) or 8 h (for nNav1.5) at 4°C. Transfer of proteins was visualized by Ponceau red staining (Sigma-Aldrich). Membranes were washed with PBS for 5 min and then ''blocked'' with PBS containing a mix of 2% bovine serum albumin (BSA) and 2% skimmed milk for 2 h at room temperature. Specific antibodies to Sigma-1 receptor or nNav1.5 were utilized to probe the blots at a concentration of 5 μg/mL in PBS with 0.1% Tween (PBST), 1% skimmed milk, and 1% BSA. The membranes were incubated with the antibodies overnight at 4°C. Blots were washed with four 5-min incubations with PBST at room temperature on an orbital shaker. Anti-rabbit horseradish peroxidase-conjugated secondary antibodies (at the manufacturers' suggested concentrations) diluted in PBST with 1% skimmed milk and 1% BSA were added for 24 h at 4°C. The blots were washed as before and then developed with an enhanced chemiluminescence Western Blot Kit (Amersham, UK).

Immunocytochemistry

Glass coverslips placed in 35 mm culture dishes were coated with poly-L-lysine (50 μg/mL). SW620 cells were seeded into the wells at 1×10^6 cells per dish and subsequently placed in a tissue culture incubator for 24 h. The cells were washed three times with Hanks buffered saline solution (HBSS) and then treated with 4% paraformaldehyde for 8 min. Following three washing steps using PBS, cells were treated with PBS containing 0.1% saponin for 10 min to permeabilize them, which was followed by another three washes with PBS. In cases where surface expression of nNav1.5 was investigated, the cells were not fixed with paraformaldehyde or permeabilized with saponin at this stage. All cells were incubated with 4% BSA in PBS for 30 min. Subsequently, antibodies to either Sigma-1 or nNav1.5 were added for 1 h at a concentration of 0.5 μg/mL, at room temperature, for fixed cells and 5 μg/mL for live cells. For controls, purified rabbit immunoglobulin G (IgG) at an equivalent concentration was used. Cells prepared for visualization of surface staining were washed three times with PBS and then fixed with 4% paraformaldehyde at room temperature for 8 min, followed by three washes with PBS at this stage. All cells were washed three times with PBS containing 0.1% Tween and then stained with the anti-rabbit antibody with fluorochromes attached, for 4 h at 4°C. After a further three washes, the cells were mounted with Vectashield (Vector Labs, Peterborough, UK) and analyzed by florescence microscopy.

Cell ELISA protocol

SW620 cells were plated into poly-L-lysine-treated 24-well plates at 0.4×10^5 cells per well. The cells were washed twice with HBSS and then either fixed with 4% paraformaldehyde for 8 min at 4°C followed by a permeabilization step with 0.1% saponin in PBS for 12 min at 4°C or for surface staining the cells were stained live without fixation or permeabilization. Cells were incubated with 3% BSA in HBSS buffer for 30 min at 4°C followed by washing three times with HBSS buffer. Subsequently, 0.5 μg/mL of the primary antibody (nNav1.5, Sigma-1 receptor, or HIF-1) in PBS with 5% BSA was added for 1 h at 37°C. However, for live stained cells, 5 μg/mL of nNav1.5 antibody was utilized. At this stage, live cells were treated with 4% paraformaldehyde for 8 min at 4°C followed by washing three times with PBS containing 4% BSA. The cells were incubated with anti-rabbit IgG Texas Red (1/50 dilution) (Sigma-Aldrich) in 1 mL of HBSS containing 4% BSA for 4 h at room temperature. Following washing of cell three times in PBS, fluorescence was quantified with a fluorescence plate reader. To take into account differences in cell proliferation/adhesion between treatments, cells were subsequently stained with crystal violet and values adjusted accordingly.

Immunoprecipitation

Protein extracts from SW620 cells were prepared utilizing an RIPA buffer containing 1% Triton-X and a mix of proteinase inhibitors (Roche). The extract was quantified and adjusted to a concentration of 1 mg/mL. Either mouse anti-Nav1.5 or a rabbit anti-Sigma-1 receptor immunoprecipitating antibody was added at a final concentration of 3 μg/mL for 16 h at 4°C. Protein-A beads were added and incubated

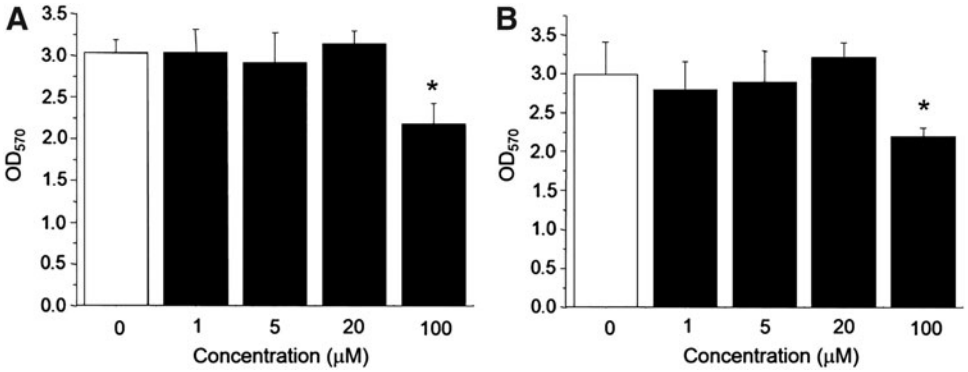

FIG. 1. Effect of Sigma-1 drugs on proliferation of SW620 cells. Cells were treated with **(A)** either SKF10047 or **(B)** 5-methyl-DMT at concentrations of 100, 20, 5, and 1 μM as indicated for 3 days. Proliferation was quantified as absorbance at 570 nm with a crystal violet assay ($n=6$). Experiments with a significant difference from the respective control treatment are marked with an asterisk ($p<0.05$). DMT, dimethyl tryptamine; OD, optical density.

with mixing for 2 h at 4°C. Spin columns (Thermo Fisher) were utilized to collect and wash the beads and the captured immunocomplexes. The beads were washed three times with PBS, and subsequently, the beads were incubated with SDS-polyacrylamide gel electrophoresis (PAGE) loading dye (Sigma-Aldrich) for 5 min. The samples were heated to 95°C for 8 min and separated using SDS-PAGE gels. Controls included performing the pull-down in the presence of a non-specific IgG antibody.

Data analysis

Means were calculated for all samples along with standard error of the means.

One-way analysis of variance test was utilized to establish statistical significance, with differences being taken as significant when $p<0.05$.

Results

Proliferation

Proliferation of SW620 cells was determined in the presence of agents with a preference for binding to Sigma-1 receptors.[15] SKF10047 or 5-methyl-DMT at concentrations of 100–1 μM was incubated with the cells (Fig. 1) and proliferation was determined after 3 days in the presence of drugs using a crystal violet assay. At concentrations of 20, 5, and 1 μM for both SKF10047 and 5-methyl-DMT, no significant difference was observed. At the higher concentration of 100 μM, significant 28% and 27% reductions in cell proliferation were recorded for SKF10047 and 5-methyl-DMT, respectively ($F=51$ $p=3.2\times10^{-5}$; $F=21$ $p=9.9\times10^{-4}$; $n=6$).

Migration

Migration plays an important role in the capability of cells to metastasize and develop secondary tumors. SKF10047 or 5-methyl-DMT was applied to SW620 cells at concentrations of 100–1 μM (Fig. 2) using a transwell assay. For SKF10047 at lower concentrations of 1 and 5 μM, small reductions in migration of 4% and 7% were recorded although these were not significant. At the higher concentrations of 20 and 100 μM, significant reductions of 13% and 20% in migration were observed ($F=13$ $p=0.017$; $F=13$ $p=0.011$; $n=6$). For 5-methyl-DMT, a significant reduction (21%) in migration was only observed at the highest concentration of 100 μM ($F=16$ $p=0.006$; $n=6$). At the other concentrations of DMT, changes in adhesion were observed with an increase of

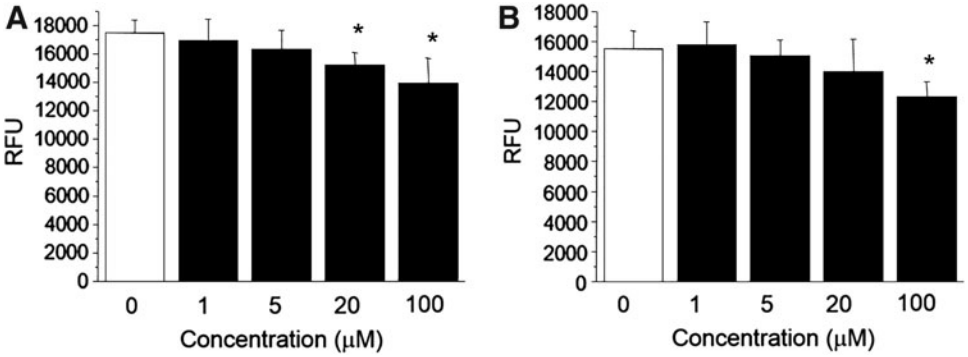

FIG. 2. Effect of Sigma-1 drugs on migration of SW620 cells. Cells were treated with SKF10047 **(A)** or 5-methyl-DMT **(B)** at concentrations of 100, 20, 5, and 1 μM for 24 h as indicated and tested using transwell migration ($n=6$). Migrated cells were labeled with calcein-A and quantified as fluorescent emission measured at 520 nm and expressed as RFU. Experiments with a significant difference from the respective control treatment are marked with an asterisk ($p<0.05$). RFU, relative fluorescent units.

adhesion of 2% at 1 μM, and decreases of adhesion of 3% and 10% for the 5 and 10 μM concentrations. However, these effects were not statistically significant.

Adhesion

Adhesion to an extracellular matrix is an important determinant for invasive cells. SKF10047 or 5-methyl-DMT at concentrations of 100–1 μM was incubated with the cells for 24 h (Fig. 3) and the attachment to collagen-coated plates was measured. SKF10047 at concentrations of 5, 20, and 100 μM reduced the adhesion of SW620 cells by 18%, 46%, and 55%, respectively ($F=6.6$ $p=0.033$; $F=47$ $p=1.3\times10^{-4}$; $F=118$ $p=4.5\times10^{-6}$; $n=5$; Fig. 3A). 5-Methyl-DMT at concentrations of 20 and 50 μM also reduced the adhesion of SW620 cells by 28% and 54% ($F=14.8$ $p=0.003$; $F=146$ $p=2.7\times10^{-7}$; $n=6$; Fig. 3B). These results are comparable with the results obtained for breast cancer cell lines MDA-MB-231 and MDA-MB-468.[14]

Sigma-1 receptor silencing in SW620 was utilized to confirm that the effects on adhesion were directly through the effect of these drugs on the Sigma-1 receptor. A significant reduction in adhesion of 30% was observed compared with the controls ($F=18.8$ $p=0.003$; $n=5$; Fig. 3C). A greater effect on adhesion was not measured when 20 μM SKF10047 was simultaneously added to Sigma-1 receptor-silenced cells (Fig. 3C). To confirm that the adhesion of cells was capable of being reduced further, TTX was utilized and a significant reduction (57%) in adhesion was recorded in comparison with all other treatments ($F=13.8$ $p=0.0046$; $n=6$). These experiments indicate that SKF10047 is directly acting on the Sigma-1 receptor.

Interaction between Sigma-1 and VGSC (nNav1.5) in SW620 cells

Sigma-1 receptors interact with Nav1.5 channel in breast cancer cell lines,[14] cardiomyocytes, or in HEK-293 cells expressing both proteins.[23] Ability of a ''blocking'' antibody to $nNa_v1.5$ to reduce the adhesion of SW620 was first determined (Fig. 4A). Using different amounts of this antibody (100–1 ng/mL), cells were incubated for 24 h and adhesion to collagen was evaluated. A reduction in adhesion was observed with 100, 20, and 5 ng/mL antibody causing a reduction of 63%, 35%, and 20%, respectively, found to be significant ($p<0.05$ for all; $n=6$; Fig. 4A). The control IgG antibody had no effect (Fig. 4A). Preincubation of the nNav1.5 antibody with the immunization peptide for the antibody blocked the antibody effect on adhesion (Fig. 4A).

The effect of combination treatments with SKF10047 (20 μM) and nNav1.5 antibody (20 ng/mL) was also measured. A significant difference in adhesion was not observed for SW620 cells treated with SKF10047 or nNav1.5 antibody alone or a combination of both ($n=6$; Fig. 4B). Silencing the Sigma-1 receptor in SW620 cells also reduced adhesion, and application of nNav1.5 antibody (20 ng/mL) to the cells had no further effect ($n=5$; Fig. 4C). These results suggested that the Sigma-1 receptor plays a significant role in the mechanism of action of nNav1.5 in these cells.

nNav1.5 and Sigma-1 receptor expression in SW620 cells

nNav1.5 protein was visualized in permeabilized cells in the cytoplasm (Fig. 5A). When the cells were not permeabilized, nNav1.5 staining was also observed (Fig. 5B). Since the

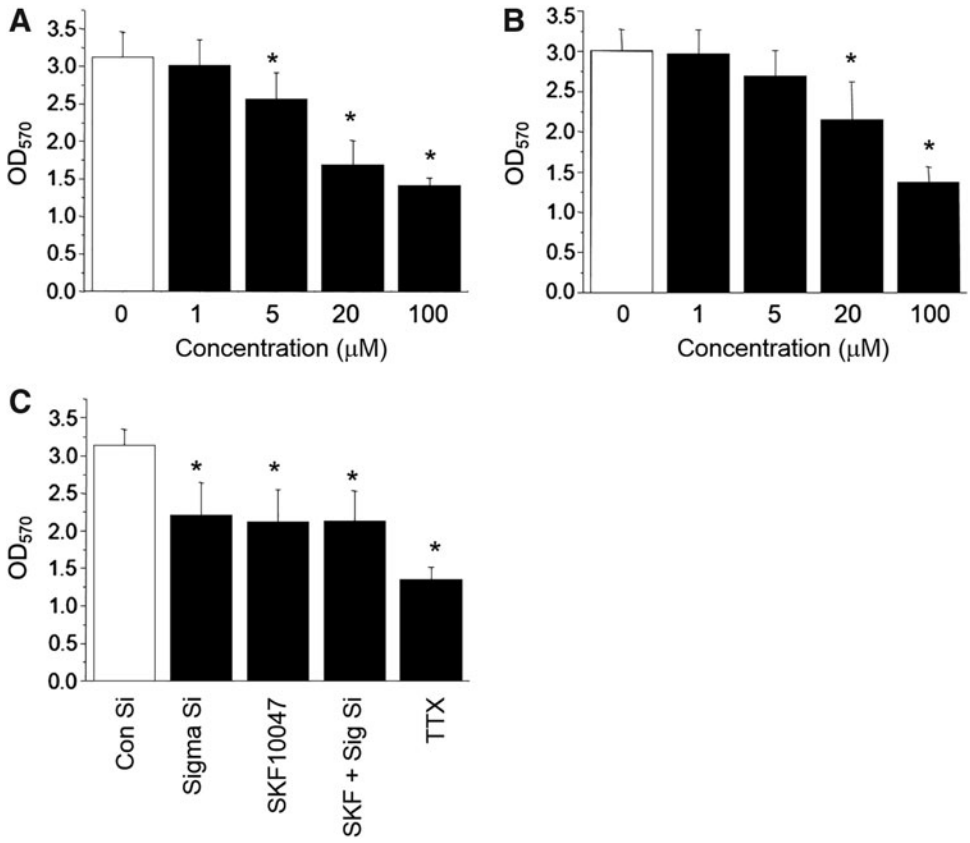

FIG. 3. Effect of Sigma-1 drugs on adhesion of SW620 cells. Cells were treated with SKF10047 **(A)** or 5-methyl-DMT **(B)** at concentrations of 100, 20, 5, and 1 μM as indicated for 24 h ($n=5$ and 6, respectively). Adhesion to collagen-coated plates was quantified as absorbance at 570 nm after staining with crystal violet. Following Sigma-1 receptor silencing, **(C)** cells were treated with SKF10047 at a concentration of 20 μM as indicated. Cellular adhesion was determined in comparison with cells transfected with a control silencing construct ($n=5$). A comparison of the effect of 10 μM TTX was made. Experiments with a significant difference from the control treatment are marked with an asterisk ($p<0.05$). TTX, tetrodotoxin.

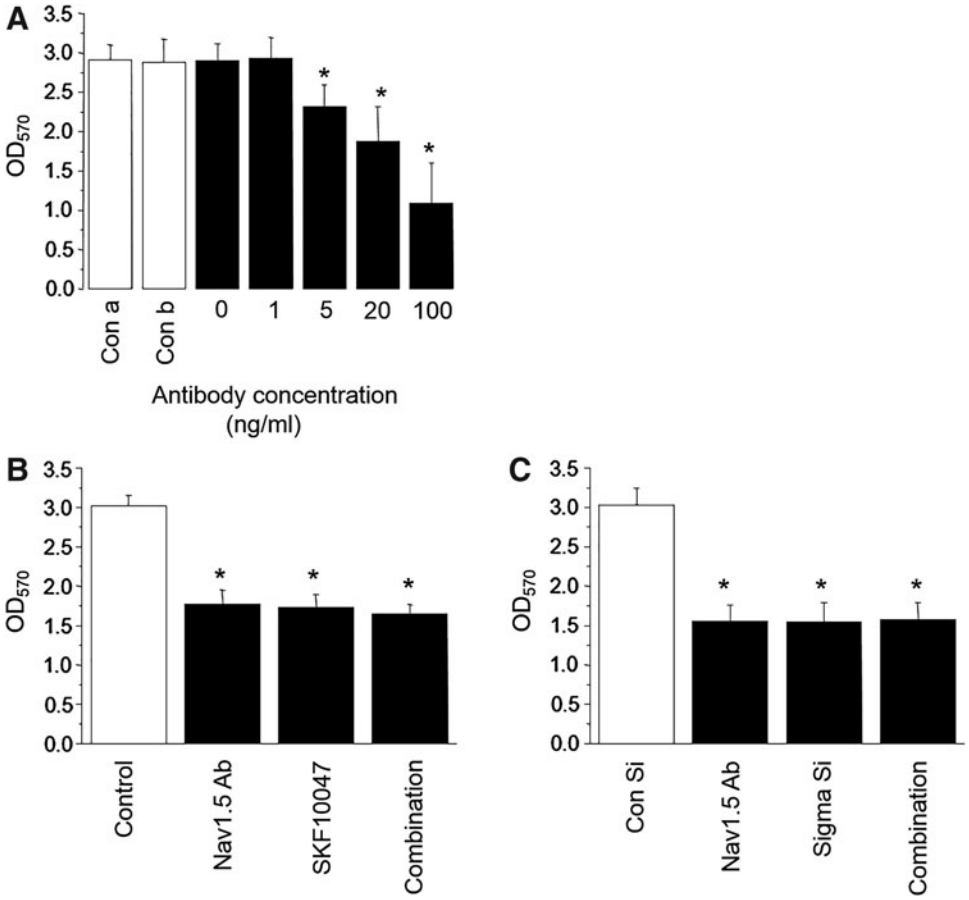

FIG. 4. Combination treatments with Sigma-1 drugs and $nNa_v1.5$ blocking antibody on adhesion of SW620 cells. The ability of a polyclonal antibody to $nNa_v1.5$ to reduce the adhesion of SW620 cells was determined **(A)**. Cells were incubated with 100, 20, 5, and 1 ng/mL of $nNa_v1.5$ polyclonal antibody for 24 h ($n=6$). Con-a consisted of 100 ng/mL of rabbit IgG in phosphate-buffered saline containing azide. Con-b consisted of 20 ng/mL of nNav1.5 antibody preincubated with 100 μg/mL of nNav1.5 peptide for 1 h at room temperature. The effect on adhesion of a combination of treatments with SKF10047 (50 μM) and $nNa_v1.5$ polyclonal antibodies (20 ng/mL) was determined following a 24-h treatment ($n=6$) **(B)**. The effect on adhesion of a combination of Sigma-1 receptor gene silencing and $nNa_v1.5$ polyclonal antibodies (20 ng/mL) was determined ($n=5$) **(C)**. Experiments with a significant difference from the respective control treatment are marked with an asterisk ($p<0.05$). Con-a, Control-a; Con-b, Control-b; IgG, immunoglobulin G.

nNav1.5 antibody binds to an extracellular domain, it is reasonable to conclude that the nNav1.5 protein is expressed in the plasma membrane and in the cytoplasm. Application of a blocking peptide to nNav1.5 reduced the observed binding of nNav1.5 antibody to the cells, indicating that the staining observed was specific to nNav1.5 ($n=5$). A Sigma-1 receptor antibody was utilized to confirm the expression of Sigma-1 receptors in SW620 cells (Fig. 5C). Expression of Sigma-1 receptors was observed although the antibody could not distinguish between different localizations of the receptor ($n=5$).

nNav1.5 expression was verified in SW620 cells by Western blotting of protein extracts. Expression was detected at the expected size of ~ 200 kDa (Fig. 6A). Sigma-1 receptor protein expression and silencing were also investigated in SW620 cells using Western blotting and an antibody to Sigma-1 receptor. Sigma-1 receptor silencing vectors were transfected into SW620 cells and after 48 h, silencing was confirmed using Western blotting in comparison with a control vector (Fig. 6B). Expression of Sigma-1 receptor was confirmed by observation of a specific band at ~ 25 kD. Levels of silencing were quantified using a cell-based ELISA in comparison with a control vector ($n=8$; Fig. 6C). Generally, Sigma-1 receptor silencing of more than 80% was obtained in experiments. Protein extracts were prepared from SW620 cells and a mouse anti-Nav1.5 antibody was used to immunoprecipitate the Nav1.5 protein and any proteins in a complex with the Nav1.5 protein. The immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes using Western blotting. Sigma-1 receptor antibodies were able to detect a band at the expected size of the Sigma-1, suggesting that Nav1.5 is in a complex with the Sigma-1 receptor ($n=4$; Fig. 6D). The experiment was carried out with the Sigma-1 receptor antibody being used to pull down the Sigma-1 receptor and associated proteins from SW620 cell protein extracts. The mouse anti-Nav1.5 antibody was able to detect the presence of Nav1.5 in the Sigma-1 receptor immunoprecipitate although we could not determine if this protein was specifically the neonatal Nav1.5 protein ($n=4$; Fig. 6E).

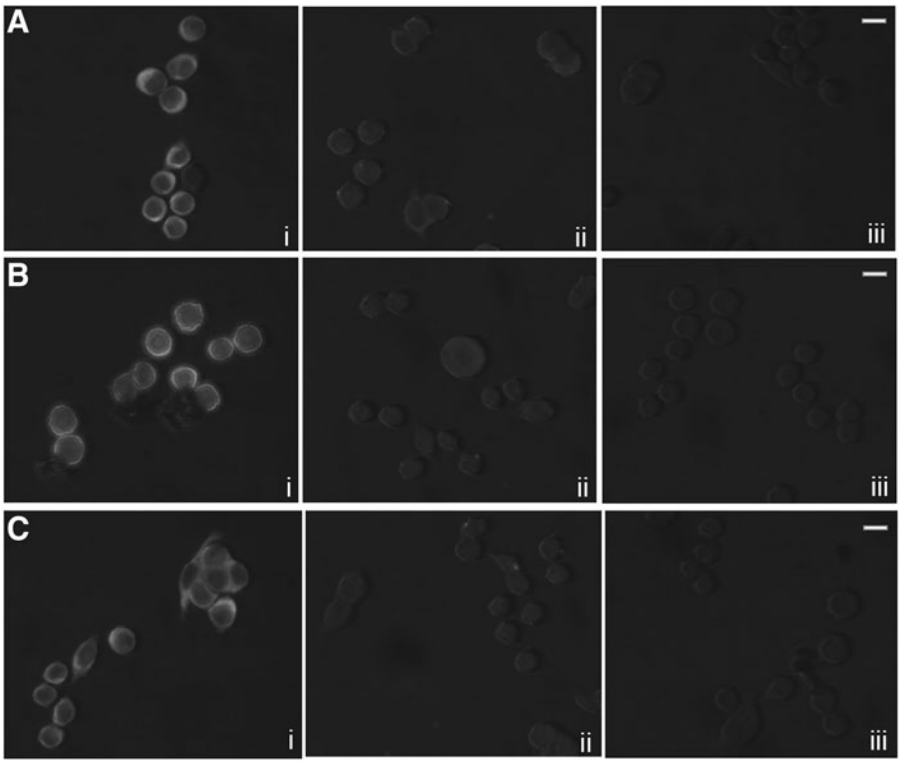

FIG. 5. Expression of $nNa_v1.5$ and Sigma-1 receptor expression in SW620 cells. **(A)** Permeabilized SW620 cells probed with antibodies to nNav1.5. (i) nNav1.5 antibody; (ii) peptide blocked antibodies; (iii) control IgG antibody. **(B)** Live stained SW620 cells probed with antibodies to $nNa_v1.5$. (i) nNav1.5 antibody; (ii) peptide blocked antibodies; (iii) control IgG antibody. **(C)** Permeabilized SW640 cells probed with antibodies to Sigma-1 receptor. (i) Sigma-1 receptor antibody; (ii) peptide blocked antibodies; (iii) control IgG antibody. Size bar represents 20 μm ($n=5$).

A combination of SKF10047 or 5-methyl-DMT and Sigma-1 receptor gene silencing was utilized and the effect on the surface expression of $nNa_v1.5$ was determined (Fig. 7A).

Treatment of SW620 cells with 50 μM SKF10047 or 5-methyl-DMT for 48 h caused 52% and 53% reductions in plasma membrane expression of $nNa_v1.5$ protein, respectively ($F=207$ $p=5.21\times10^{-8}$; $F=170$ $p=1.32\times10^{-7}$). Similar results were obtained from Sigma-1 receptor-silenced cells with a reduction in surface expression of nNav1.5 of 53% compared with a gene silencing control ($F=611$ $p=2.68\times10^{-10}$). Treatment of Sigma-1-silenced cells with SKF10047 did not produce any significantly greater effect. It was concluded that the Sigma-1 receptor increased the plasma membrane expression of nNav1.5 in SW620 cells and this was inhibited with Sigma drugs or gene silencing. Effect of the above treatments on the total expression of nNav1.5 was also verified. No significant differences in total expression of nNav1.5 were observed (Fig. 7B).

Effect of hypoxia on Sigma-1 receptors

SW620 cells were cultured under normoxic or hypoxic conditions for 24 h. Subsequently, the level of Sigma-1 receptor protein was ascertained using a cell-based ELISA with antibodies to the Sigma-1 receptor. The results (Fig. 8A) indicated that under hypoxic conditions, Sigma-1 protein levels increased by more than sixfold, which was found to be significant ($F=188$ $p=1.62\times10^{-9}$; $n=8$). To verify that hypoxia had been induced, the cells were also stained for Hif-1 alpha expression. The data (Fig. 8B) indicated that Hif-1 alpha was increased 3.71-fold, which was significant ($F=12$ $p=0.0034$; $n=8$). Cells were also tested for viability, but no significant change in viability was observed between normoxic and hypoxic cells (data not shown). Some changes in proliferation were observed between normoxic and hypoxic conditions ($\sim12\%$ overall) and, as such, all fluorescence values were adjusted based on the change in proliferation. Using the same assay, SW620 cells were also tested for the levels of total nNav1.5 protein. The data (Fig. 8C) indicated a significant 2.08-fold increase in total nNav1.5 protein under hypoxic conditions ($F=43$ $p=1.27\times10^{-5}$; $n=8$). This increase was not significantly affected by the application of SKF10047 for 24 h. A significant increase in surface nNav1.5 (by 2.02-fold) was also recorded (Fig. 8D) when comparing normoxic and hypoxic conditions ($F=43$ $p=1.26\times10^{-5}$; $n=7$). Interestingly, application of SKF10047 for 24 h resulted in a significant decrease in nNav1.5 levels ($F=20$ $p=4.81\times10^{-4}$; $n=8$) approaching the level found under normoxic conditions.

Discussion

It has been well documented that Sigma-1 receptor drugs are capable of changing the invasive aspects of cancer cells (for a review, Aydar et al.[15]), however, the exact mechanisms have remained unclear. Another well-documented aspect of Sigma-1 receptors is their ability to interact with a number of different ion channels.[21–23] In MDA-MB-231 cells, VGSC current density was modulated by Sigma-1 receptors.[24] Previously, it was described that modulation of Sigma-1 receptors in breast cancer cell lines can affect their invasive potential and that this effect was mediated via expression of nNav1.5. VGSC (nNav1.5) upregulation occurs in metastatic breast and colon cancer cells and this promotes the cell invasive properties.[4,6,13,28]

Sigma-1 receptors form a complex with VGSCs in breast cancer cells,[24] which was further substantiated by

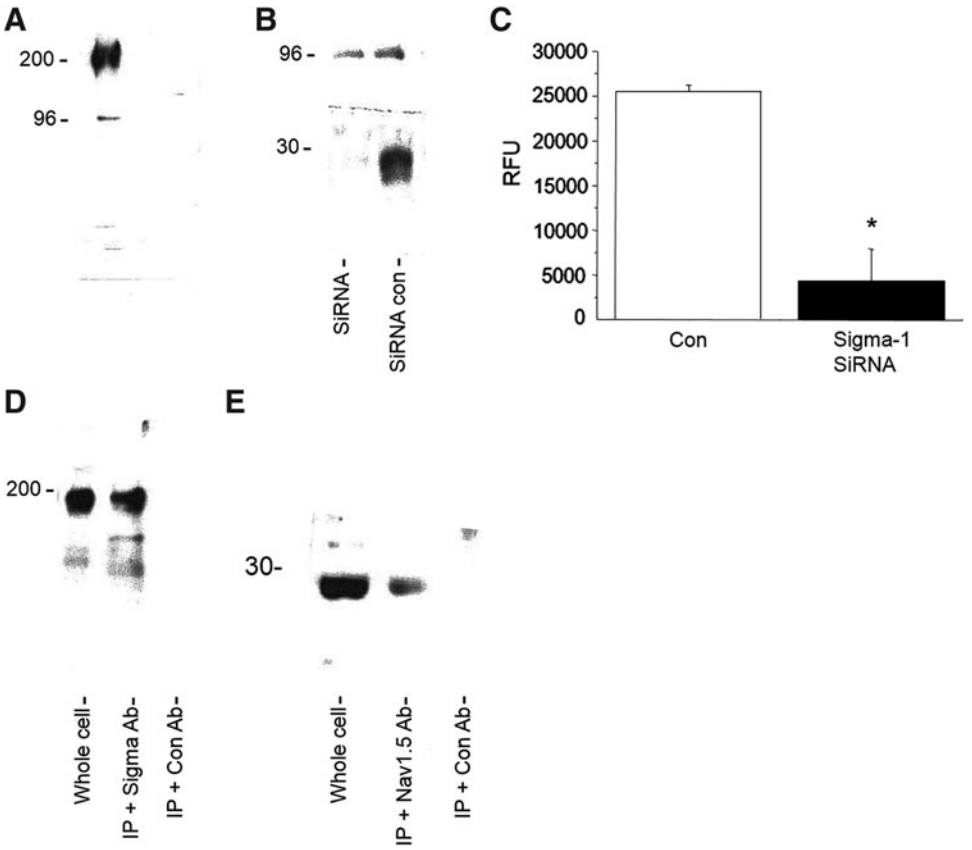

FIG. 6. Expression of nNa$_v$1.5 and Sigma-1 receptor in SW620, and immunoprecipitation between Na$_v$1.5 and Sigma-1 receptor. **(A)** Western blot of SW620 cells probed with antibodies to nNav1.5 and actinin ($n=4$). Position of size markers at 200 and 96 kDa is shown. **(B)** Western blot of SW620 cells probed with antibodies to Sigma-1 receptor and actinin ($n=4$). Cells are transfected with a Sigma-1 receptor silencing vector or a control plasmid. Position of size markers at 96 and 30 kDa is indicated. **(C)** Quantification of gene silencing of Sigma-1 receptors in SW620 cells. Cell-based ELISA data from Sigma-1 receptor gene-silenced and control cells ($n=8$). **(D)** Immunoprecipitation of Nav1.5 by Sigma-1 receptor antibodies. Whole-cell extract, immunoprecipitation with Nav1.5 antibodies, and immunoprecipitation with a control IgG antibody are shown ($n=3$). Position of size markers at 200 kDa is shown. **(E)** Immunoprecipitation of Sigma-1 receptor by Nav1.5 antibodies. Whole-cell extract, immunoprecipitation with Nav1.5 antibodies, and immunoprecipitation with a control IgG antibody are shown ($n=3$). Position of size markers at 30 kDa is shown. ELISA, enzyme-linked immunosorbent assay. Experiments with a significant difference from the respective control treatments are marked with an asterisk ($p<0.05$).

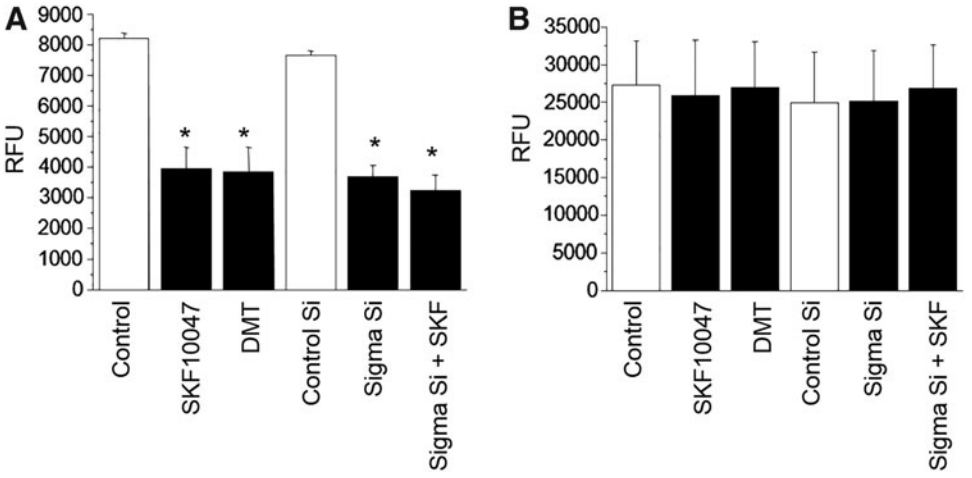

FIG. 7. Effect of Sigma-1 receptor on surface expression of nNav1.5 in SW620 cells. **(A)** Cells were treated with SKF10047 or DMT at a concentration of 50 μM for 48 h or the Sigma-1 receptor was gene silenced and subsequently the surface expression of nNav1.5 was determined by probing with an antibody to nNav1.5 in live cells ($n=12$). **(B)** In addition, combination treatments with both SKF10047 and Sigma-1 gene silencing were carried out. Surface expression of nNav1.5 was quantified using a fluorescent plate reader and expressed as RFU. Experiments with a significant difference from the respective control treatments are marked with an asterisk ($p<0.05$). In addition, the total expression of nNav1.5 in SW620 cells under the same experimental conditions was verified ($n=12$).

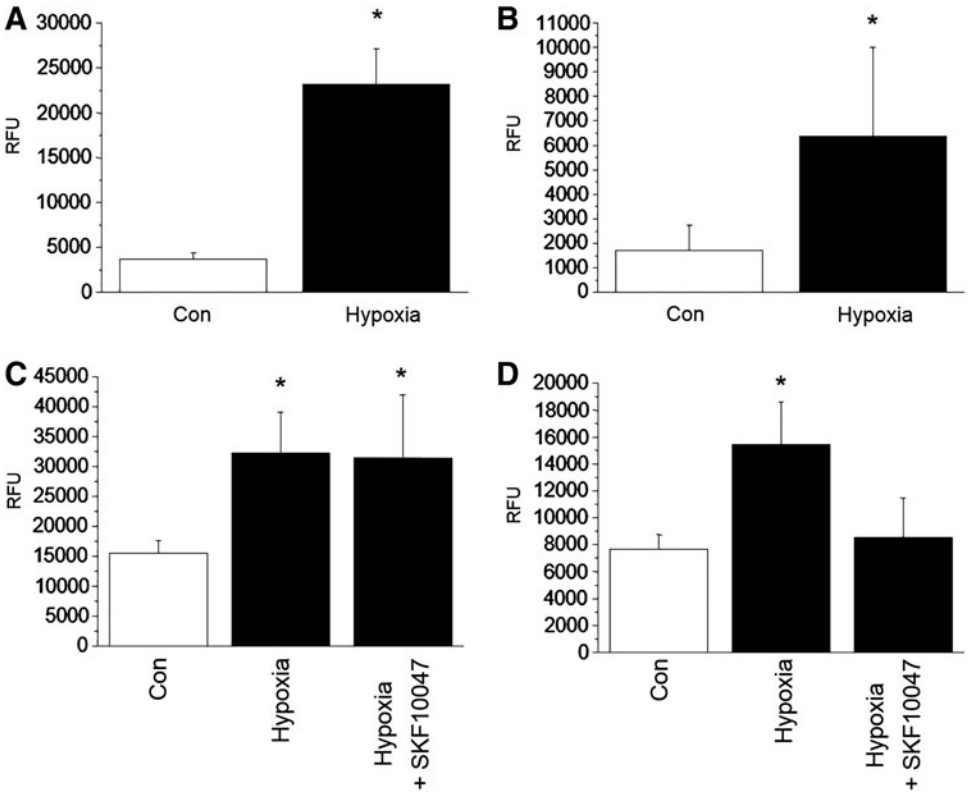

FIG. 8. Effect of hypoxia on Sigma-1 receptor expression and surface expression of nNav1.5 in SW620 cells. SW620 cells were transferred to a hypoxia incubator for 24 h and the levels of Sigma-1 receptor protein **(A**; $n=8$), Hif-1 **(B**, $n=8$), and total nNav1.5 **(C**; $n=7$) were quantified and compared with cells under normal culture conditions using a cell-based ELISA. In the case of nNav1.5, cells under hypoxic conditions were treated with SKF10047 and compared with hypoxic cells without SKF10047. In similar experiments, **(D)** cells were also live-stained and tested for the presence of surface nNav1.5. Following all experimental conclusions, the cells were stained with a crystal violet assay to account for differences in cell proliferation and potential loss of cells due to altered adhesion. Fluorescence values were adjusted accordingly. Experiments with a significant difference from the respective control treatments are marked with an asterisk ($p<0.05$).

experiments indicating functional complexes existing between Sigma-1 receptors and VGSCs, which are capable of increasing the plasma membrane expression of VGSCs and increasing the invasive properties of breast cancer cells at least by modulating the adhesiveness of cells to the extracellular matrix.[14]

We aimed in this study to investigate a possible functional link between Sigma-1 receptors and nNav1.5 in colon cancer cells and to investigate whether the effects of Sigma-1 drugs in altering the invasive behavior of colon cancer cells occur by an interaction with nNav1.5. As reported previously,[6] colon cancer cells express nNav1.5 predominantly. Expression of nNav1.5 was documented in this study by both Western blotting and immunostaining and was found both in the cytoplasm and at the plasma membrane surface due to the use of an antibody that binds to an extracellular domain of nNav1.5. Expression of the Sigma-1 receptor was documented by both Western blotting and immunostaining. However, the antibody utilized was unable to distinguish the exact cellular localization in these cells.

An effect of Sigma-1 drugs on proliferation was only found at higher concentrations of Sigma-1 drugs. We speculate that at these higher drug concentrations, the Sigma-2 receptor may also be affected. In addition, only a modest reduction in migration was observed, although it is possible that pretreatment of the cells with Sigma drugs may increase these values. We found that Sigma-1 receptor drugs were able to significantly alter the adhesiveness of metastatic colon cancer cell line SW620 to collagen, an important factor in invasiveness. Gene silencing of the Sigma-1 receptor significantly decreased the adhesion of these cells. This observed effect on adhesion was proposed to be via the drugs acting on the Sigma-1 receptor as no further effect on adhesion was observed when the Sigma-1 receptor was silenced or Sigma-1 drugs were applied. We observed that application of an antibody that binds and blocks the nNav1.5 channel in breast cancer cells and reduces the adhesion of those cells was also capable of reducing the adhesion of SW620 cells, further documenting the importance of nNav1.5 in the invasive properties of these cells, which was recently documented.[6]

The Sigma-1 receptor was found to be in a complex with Nav1.5 in these cells using immunoprecipitation, although in these particular experiments it was not possible to distinguish between the adult form of Nav1.5 and the neonatal form. Previously, it was demonstrated that the Sigma-1 receptor binds directly to Nav1.5 with fourfold symmetry, suggesting that the Sigma-1 receptor interacts with the transmembrane regions of its partners. Also, two other Sigma-1 ligands, haloperidol and (+)-pentazocine, disrupted this interaction both *in vitro* and in living cells.[24]

Sigma-1 receptor gene silencing or treatment with Sigma-1 receptor drugs resulted in a clear reduction in the surface expression of nNav1.5 although neither treatment caused a reduction in the total amount of nNav1.5. Hypoxia is known to be a major driving force in the switch from cells becoming invasive. The effect of hypoxia on Sigma-1 receptor expression in SW620 has been previously reported.[29] Our results are in agreement indicating a significant rise in Sigma-1 receptor expression under hypoxic conditions. The effect of hypoxia on nNav1.5 total expression was determined and we observed an approximate twofold increase in nNav1.5 expression. Similar experiments were carried out,[6] although in this case the experimenters did not see an increase in nNav1.5. However, our experiments utilized a cell-based assay that is more likely to measure small changes in protein levels and utilizes a higher number of repeat experiments.

Application of Sigma-1 drugs did not affect the total expression of nNav1.5 under hypoxic conditions. However, it did effect the expression of surface expressed nNav1.5, lowering the level of surface nNav1.5 to the levels seen in normoxic cells. Our interpretation is that under hypoxic conditions, Sigma-1 receptor expression is upregulated to promote survival of the cell. One of the ''client'' proteins of the Sigma-1 receptor is the nNav1.5 protein, which is also upregulated. The interaction between the Sigma-1 receptor and nNav1.5 results in increased surface expression of nNav1.5, which increases the invasiveness of the cells via an increase in adhesion and migration. The Sigma-1 receptor is hypothesized to be ''silent'' under normal conditions, and only comes to play on cell physiology when the cell is under ''stress'' such as upon disease.

In this case, Sigma-1 receptors act as a ''chaperone'' trafficking ion channels to the plasma membrane affecting the electrical plasticity of the cell.[25] This interaction likely occurs early in invasion as VGSC expression affects a network of genes controlling invasiveness in colon cancer.[7] There is evidence to support this idea as a protein interaction between Sigma-1 receptors and $K_v1.2$ channels was induced upon cocaine exposure. This resulted in a redistribution of both proteins to the plasma membrane.[30]

We speculate that hypoxia-driven increases in levels of the Sigma-1 receptor as reported previously[29] in colon cancer cell lines may result in an increase of the plasma membrane expression of nNav1.5 by the Sigma-1 receptor acting as a chaperone protein. Coupled with this is the previously demonstrated hypoxia-driven upregulation of nNav1.5 in SW620 cells,[6] culminating in colon cancer cells with an increased invasive potential, possibly to ''escape'' their hypoxic conditions resulting eventually in metastasis. Importantly, we acknowledge that these experiments have been performed on one model cell line. It would be essential in the future to investigate this mechanism further in a variety of other colon cancer cell lines.

Conclusions

The Sigma-1 receptor appears to play an important role in VGSC delivery to the plasma membrane and this has clinical implications, given the prominent involvement of nNav1.5 in metastatic disease.[11,31]

Author Disclosure Statement

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