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Loss of gap junctional intercellular communication in rat lung epithelial cells exposed to quartz particles

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ABSTRACT

Chronic inhalation of quartz particles has been implicated in lung diseases including silicosis and cancer. The aim of this study was to investigate whether quartz particles affect gap junctional intercellular communication (GJIC) in rat lung epithelial cells (RLE-6TN). Here, we demonstrate that exposure of RLE-6TN cells to subtoxic doses of DQ12 standard quartz resulted in an up to 55% reduction of GJIC, as determined in a dye transfer assay. We show that connexin-43 (Cx43) is the major connexin responsible for intercellular communication in these lung epithelial cells and that exposure to quartz particles induces a significant internalization of Cx43. Downregulation of GJIC was attenuated by *N*-acetyl cysteine, suggesting the involvement of reactive oxygen species and/or cellular thiol homeostasis in the regulation of GJIC. Furthermore, an inhibitor of activation of extracellular signal-regulated kinases prevented the loss of GJIC in cells exposed to DQ12 quartz, although no direct phosphorylation of Cx43 upon exposure to DQ12 was detected.

Introduction

Inhalation of quartz particles is known to cause the development of lung pathologies such as silicosis, including fibrosis and inflammatory processes, and lung cancer [1]. The loss of intercellular communication via gap junctions was described as an early characteristic in the development of cancer cells [2] and to be caused by inflammatory mediators, including reactive oxygen species [3]. Gap junctions consist of groups of intercellular channels built of connexin molecules, with each of the adjacent cells contributing a connexin-hexamer half channel to form a gap junctional channel [4]. Gap junctional intercellular communication (GJIC) results from the regulated diffusion of compounds of low molecular mass (less than approx. 1 kDa) between cells and is controlled at the levels of connexin (Cx) expression, posttranslational Cx modification, such as phosphorylation, and subcellular Cx distribution [5].

The purpose of this study was to test whether a change in GJIC could serve as an early measure of adverse effects of particle exposure of lung alveolar cells, and to test whether exposure to carcinogenic quartz particles results in a detectable loss of GJIC, as would be expected from a carcinogen.

We here demonstrate that cultured rat lung alveolar epithelial cells communicate via gap junctional channels consisting of Cx43. This GJIC is drastically attenuated upon exposure to subcytotoxic doses of standard DQ12 quartz particles. The loss of GJIC, although not coinciding with a detectable change in Cx43 phosphorylation was attenuated by inhibition of activation of Cx43 kinases, extracellular signal regulating kinases (ERK) 1 and 2, and it correlated with a significant alteration of subcellular distribution of Cx43.

Materials and methods

Particles and particle preparation. Standard DQ12 quartz (Dörentruper Quarz, batch 7) with particles of <5 μm in diameter were supplied by Institut für umweltmedizinische Forschung (IUF), Duesseldorf, Germany [6]. Stock suspensions of particles (1 mg/ml) were prepared in PBS by sonication for 60 min. The suspensions were diluted in PBS and applied to adherent cells in cell culture media to yield a final concentration of 10 μg/cm^2.

Cell culture and transfections. RLE-6TN rat lung epithelial cells derived from alveolar type II cells were provided by Dr. K. Driscoll, Procter & Gamble, Cincinnati, USA and were cultivated at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 and held in Ham's F-12 (Sigma, Deisenhofen, Germany, or PAA, Pasching, Austria) supplemented with (final concentrations) 10% (v/v) fetal calf serum (FCS "gold"; BioWest, Frickenhausen, Germany, or PAA), 2 mM L-glutamine or 1% (v/v) of Glutamax (Invitrogen), and penicillin/streptomycin. For transfections with siRNA cells were seeded on 3 cm dishes, followed 24 h later by transfection using oligofectamine transfection reagent (Invitrogen). The employed control

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(5′-ugguuuacaugucgacuaa-3′) and Cx43 (Smart pool: 5′-caacaaccuggcugcgaaa-3′, 5′-ugauugaaaugucgaguua-3′, 5′-cgugaagggaagaagcgau-3′, 5′-uuacugagauucugcgaua-3′) siRNAs were obtained from Dharmacon/ThermoFisher (Epsom, UK).

Determination of gap junctional intercellular communication (GJIC). Cells were grown to approx. 80% confluence on 3 cm dishes and held in serum-free medium for 20 h, followed by exposure to DQ12 particles for up to 24 h. GJIC was determined by microinjecting the fluorescent dye Lucifer Yellow CH (Sigma; 10% (w/v) in 0.33 M LiCl) into selected cells by means of a micromanipulator and a microinjector system (Eppendorf, Hamburg, Germany). One minute after injection, fluorescent cells surrounding the cells loaded with the dye were counted and taken as a measure of GJIC. Ten individual cells were loaded with dye per dish and means of the numbers of fluorescent neighboring cells were calculated [7].

Western blotting, immunocytochemistry. All immunochemical assays were described earlier [8]. For Western blotting, cells were lysed in 0.5% (w/v) sodium dodecyl sulfate and protein concentrations determined in a bicinchoninic acid (BCA)-based protein assay (Pierce/Thermo Scientific, Bonn, Germany), followed by addition of 2× SDS–PAGE buffer [125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 100 mM DTT, 0.2% (w/v) bromophenol blue], brief sonication and 5 min of incubation at 95 °C. Samples were applied to SDS–polyacrylamide gels of 10% (w/v) acrylamide, followed by electrophoresis and blotting. Immunodetections were performed using the following antibodies (supplier; dilution): rabbit polyclonal anti-Cx43 (Sigma; 1:1500), mouse monoclonal anti-GAPDH (Chemicon, Temecula, CA, USA; 1:1000). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were used as secondary antibodies (Amersham Pharmacia Biotech/GE Healthcare). All antibody incubations were in 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST).

For immunocytochemistry, cells were grown on glass coverslips and treated with siRNA as described above, if required. Following the respective experimental treatments, cells were washed with PBS and fixed either for 10 min in ice-cold methanol or for 30 min with 4% of formaldehyde, and washed three times with PBS. Non-specific binding sites were blocked for 1 h at room temperature with 3% (v/v) normal goat serum (Life Technologies, Rockville, MD) diluted in PBS containing 0.3% (v/v) Triton X-100. For detection of Cx43, cells were incubated with polyclonal rabbit anti-connexin-43 (1:1500) diluted in PBS containing 1% (v/v) goat serum overnight at 4 °C. Antibodies were removed and cells washed three times with PBS, followed by incubation with an Alexa 594-coupled goat anti-rabbit IgG (H + L; 1:800) for 1 h at 37 °C. Afterwards, cells were washed three times and embedded with ProLong Gold/DAPI mounting medium (Invitrogen). Fluorescence microscopic images were taken with an AXIOVERT 200 M microscope (Zeiss, Oberkochen, Germany).

Results and discussion

GJIC between rat alveolar type II epithelial cells is mediated by Cx43

Alveolar type II epithelial cells express several connexin isoforms, including Cx26, Cx32, and Cx43 [9]. In order to test for the

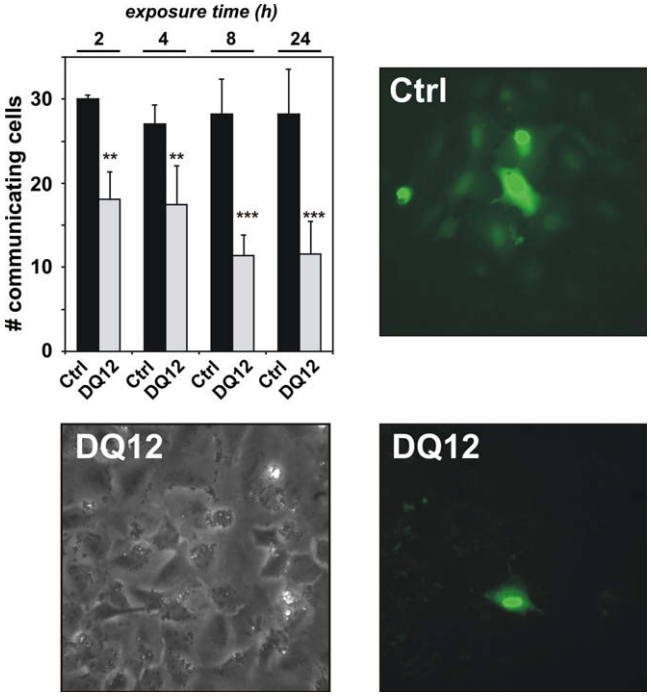

Fig. 2. GJIC in rat lung epithelial cells exposed to DQ12. Cultured RLE-6TN cells were grown to near confluence and exposed to DQ12 (10 μg/cm^2) for the given periods of time, followed by determination of GJIC by dye transfer analysis. Data are given as means ± SD (n = 3). ANOVA with LSD post-test was used for the determination of statistical significance of differences between treatment (DQ12, gray bars) and the respective control (Ctrl, black bars) groups. $^{**}P < 0.01$, $^{***}P < 0.001$. Representative images of cultured cells not exposed (Ctrl) or exposed to DQ12 are given that were taken about 60 s after Lucifer Yellow had been injected into one cell.

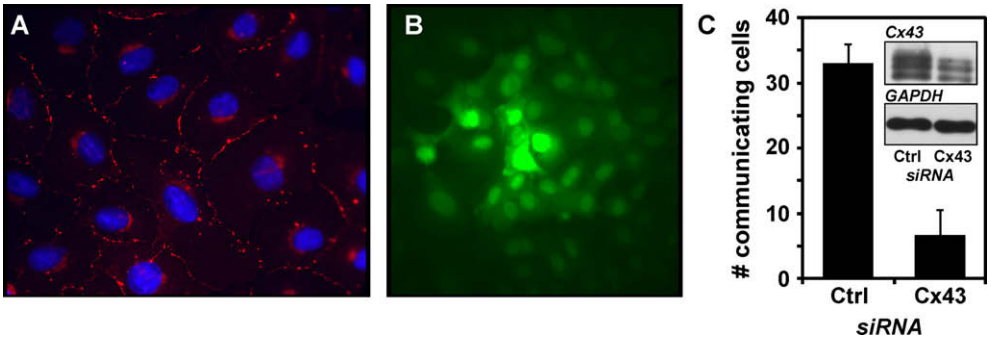

Fig. 1. Cx43 expression and gap junctional intercellular communication (GJIC) in rat lung epithelial cells. (A) Cultured RLE-6TN cells were tested for Cx43 expression (red) by immunohistochemistry. Nuclei were stained with DAPI (blue). (B) Determination of GJIC in RLE-6TN cells by microinjection and analysis of lateral diffusion of the fluorescent dye Lucifer Yellow. A representative image of cultured cells is given that was taken about 60 s after Lucifer Yellow had been injected into one cell. (C) Determination of GJIC by dye transfer analysis in RLE-6TN cells 48 h after transfection of Cx43-specific or control (Ctrl) siRNA. Data are means ± standard deviation (SD) (n = 4). Inset: Western blot analysis of Cx43 levels in siRNA-treated cells 48 h after transfection. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were analyzed to control for equal protein loading of the gel. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

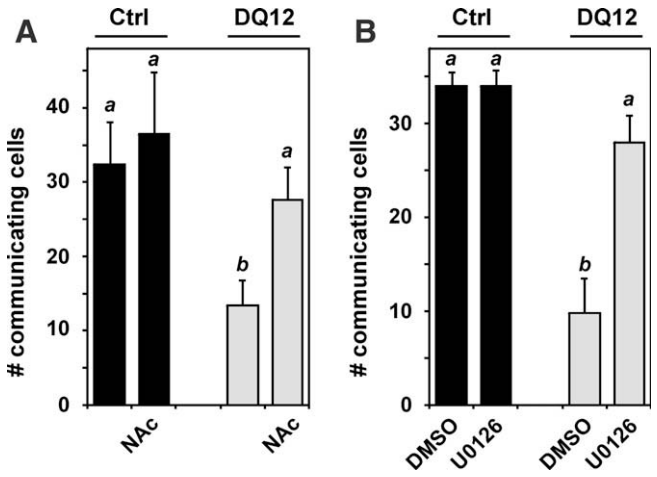

Fig. 3. Role of ERK signaling in particle-induced loss of GJIC. RLE-6TN cells were grown to near confluency and incubated (A) in the presence of 5 mM *N*-acetyl cysteine (NAc) overnight as noted or (B) in the presence of the MEK-1/2 inhibitor U0126 (10 μM) or the same volume of DMSO (solvent control) for 30 min prior to change of media and exposure of cells to DQ12 (10 μg/cm^2) for 4 h. In (B), exposure was in the continued presence of U0126 or DMSO. GJIC was then determined by dye transfer analysis. Data are given as means ± SD ($n = 3$). ANOVA with LSD post-test was used for the determination of statistical significance of differences between treatment groups. $P < 0.05$ was selected as the level of significance. Treatment groups are significantly different from each other if no labeling letter (*a* or *b*) is shared between groups.

capability of RLE-6TN rat lung epithelial cells to communicate via gap junctions and to test for the involvement of the most widely expressed connexin, Cx43, cells were analyzed for Cx43 expression and for GJIC. RLE-6TN cells express Cx43, which was detected by immunocytochemistry in spots lining the cell membranes (Fig. 1A), and they are capable of GJIC, as judged from the diffusion of Lucifer Yellow from a cell that was loaded with dye by microinjection to its neighbors (Fig. 1B). Depletion of Cx43 using a Cx43-specific siRNA further demonstrated that GJIC in cultured RLE-6TN cells is almost entirely due to gap junctions built of Cx43: GJIC is lower in cultures depleted of Cx43 (Fig. 1C), with the extent of loss (approx. 80%) similar to the extent of Cx43 depletion achieved (see Western blot, Fig. 1C, inset).

Loss of GJIC in rat lung epithelial cells exposed to DQ12 fine silica particles

Exposure of RLE-6TN cells to standard DQ12 particles that were extensively characterized previously [6] caused a significant reduction of GJIC (Fig. 2). The observed loss of GJIC was persistent and was detected for at least 24 h. The dose of 10 μg/cm^2 of DQ12 that was chosen for exposure was subcytotoxic as determined microscopically (Fig. 2) and by standard toxicity assays employing the tetrazolium salt WST-1 (data not shown).

On the mode of downregulation of GJIC by exposure to quartz particles

Silica particle action on target cells has been demonstrated to cause the formation of reactive oxygen species (ROS) [6,10,11]. As ROS are known modulators of GJIC via stimulation of extracellular signal-regulated kinases (ERK) and ERK-dependent Cx43 phosphorylation [7,12], and as DQ12 was demonstrated to cause ERK phosphorylation in murine lung epithelial cells [13] and in RLE-6TN cells (C. Albrecht, unpublished results), we tested for the effects of preincubation of cells with a cell permeant thiol and general antioxidant, *N*-acetyl cysteine (NAc), and of pretreatment with an inhibitor of ERK activation, U0126, on DQ12-induced modulation of GJIC. As depicted in Fig. 3, both preincubation with NAc and with U0126 prevented the loss of GJIC that was achieved by exposure to DQ12 quartz particles, suggesting the involvement of ROS and ERK in modulation of GJIC.

In order to test the resulting hypothesis that the loss of GJIC is due to an ERK-dependent connexin phosphorylation which then results in a change of subcellular distribution of connexin molecules, we analyzed Cx43 distribution in RLE-6TN cells by immunocytochemistry and tested for modulations of Cx43 levels and phosphorylation by Western blotting. Whereas in control cells Cx43 molecules were detected primarily in the cell membranes lined up at cell/cell interfaces, Cx43 appeared to be located primarily intracellularly in cells exposed to DQ12 (Fig. 4A). The Cx43 localization pattern realized under quartz exposure conditions reminds of that elicited in cells exposed to the tumor promotor, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which was previously described to cause connexin internalization in different cell types [7,14]; similarly, TPA caused Cx43 internalization in rat lung

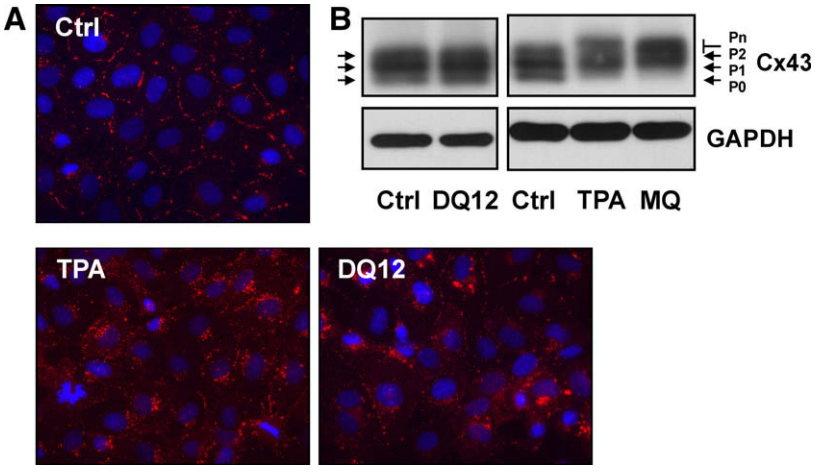

Fig. 4. Cx43 levels and subcellular localization in particle-exposed cells. (A) Immunocytochemical analysis of Cx43 (red) distribution in RLE-6TN cells exposed to DQ12 (10 μg/cm^2) for 4 h. Nuclei were stained with DAPI. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 100 nM) was taken as control for Cx43 internalization. (B) RLE-6TN cells were grown to near confluence and exposed to DQ12 (10 μg/cm^2) for 4 h or to TPA (100 nM) or menadione (MQ, 50 μM) for 30 min, followed by lysis and Western analysis of Cx43 and GAPDH levels as well as changes in electrophoretic mobilities of Cx43 bands. P0, P1, P2, and Pn denote different forms of Cx43 with varying extent of phosphorylation, with P0 the unphosphorylated form. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

epithelial cells (Fig. 4A). TPA was also reported to cause phosphorylation of Cx43. In fact, analysis of Cx43 levels and electrophoretic mobility patterns by SDS–PAGE and Western blotting revealed that TPA, while not affecting Cx43 levels, induced a significant shift in electrophoretic mobility of Cx43 relative to control (Fig. 4B). Several different Cx43 bands were detectable by Western blotting that are due to various differentially phosphorylated forms of Cx43. An attenuation of electrophoretic mobility, as seen with TPA, is based on the enhanced phosphorylation of Cx43. In addition to TPA, which causes protein kinase C-dependent [15] and ERK-dependent [14] Cx43 phosphorylation, we tested for the effect of menadione on Cx43 phosphorylation. Menadione, a redox cycling and alkylating agent causing oxidative stress, was previously described by us to cause ERK-dependent Cx43 phosphorylation [7,16]. Again, the slower migration of phosphorylated Cx43 in the polyacrylamide gels was clearly visible in Western blots (Fig. 4B). In contrast, no phosphorylation-induced shift of Cx43 electrophoretic mobility was detected in lysates from cells exposed to DQ12. Similarly, Cx43 levels were not changed in cells exposed to DQ12 (Fig. 4B).

Conclusions

We here demonstrate that exposure of rat lung epithelial cells that communicate via Cx43-based gap junctions (Fig. 1) to standard DQ12 quartz particles causes a dramatic loss of GJIC (Fig. 2). This loss is attenuated by *N*-acetyl cysteine and an inhibitor of ERK activation (Fig. 3) but does not coincide with a detectable phosphorylation of Cx43 molecules (Fig. 4B). Rather, Cx43 is internalized and depleted from the cell membrane (Fig. 4A). This pattern of an ERK-dependent but apparently phosphorylation-independent loss of Cx43-mediated GJIC was previously described for cells exposed to the anthraquinone chemotherapeutic doxorubicin that, like DQ12 (Fig. 3), causes the formation of ROS and the activation of ERK in cells [17]. Although this does not fully explain the mechanism of loss of GJIC in cells exposed to quartz particles, it at least allows for a classification of the DQ12 effects as "doxorubicin-like". It was recently demonstrated [18] that Cx43 internalization may be elicited by loss of the adherens junction associated protein β-catenin. It is further known that oxidative stress may cause redistribution of β-catenin/adherens junction protein complexes as a consequence of stress-induced phosphorylation [19]. Therefore, we hypothesize that the loss of GJIC and the internalization of Cx43 elicited by exposure to DQ12 may be brought about by interference of the particles with adherens junction integrity.

Gap junctional intercellular communication (GJIC) has been hypothesized to play a crucial role in the regulation of carcinogenesis. In fact, it was recently suggested that determination of GJIC may prove suitable as a standard screening assay for nongenotoxic carcinogens [20]. We have provided an example supporting this proposal: crystalline silica was categorized carcinogenic [21] and was here shown to indeed strongly impair GJIC.

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