



Silica nanoparticles induce endoplasmic reticulum stress response and activate mitogen activated kinase (MAPK) signalling



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ABSTRACT

Humans may be exposed to engineered silica nanoparticles (SiO₂-NPs) but potential adverse effects are poorly understood, in particular in relation to cellular effects and modes of action. Here we studied effects of SiO₂-NPs on cellular function in human hepatoma cells (Huh7). Exposure for 24 h to 10 and 50 μ g/ml SiO₂-NPs led to induction of endoplasmic reticulum (ER) stress as demonstrated by transcriptional induction of *DNAJB9*, *GADD34*, *CHOP*, as well as CHOP target genes *BIM*, *CHAC-1*, *NOXA* and *PUMA*. In addition, CHOP protein was induced. In addition, SiO₂-NPs induced an inflammatory response as demonstrated by induction of TNF- α and *IL-8*. Activation of MAPK signalling was investigated employing a PCR array upon exposure of Huh7 cells to SiO₂-NPs. Five of 84 analysed genes, including *P21*, *P19*, *CFOS*, *CJUN* and *KSR1* exhibited significant transcriptional up-regulation, and 18 genes a significant down-regulation. Strongest down-regulation occurred for the proto-oncogene *BRAF*, *MAPK11*, one of the four p38 MAPK genes, and for *NFATC4*. Strong induction of *CFOS*, *CJUN*, *FRA1* and *CMYC* was found after exposure to 50 μ g/ml SiO₂-NPs for 24 h. To analyse for effects derived from up-regulation of TNF- α , Huh7 cells were exposed to SiO₂-NPs in the presence of the TNF- α inhibitor saquinone, which reduced the induction of the TNF- α transcript by about 50%. These data demonstrate that SiO₂-NPs induce ER stress, MAPK pathway and lead to inflammatory reaction in human hepatoma cells. Health implications of SiO₂-NPs exposure should further be investigated for a risk assessment of these frequently used nanoparticles.

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1. Introduction

Engineered silica nanoparticles (SiO₂-NPs) are among the most popular nanoparticles. They are applied in industrial manufacturing, and applications including cosmetics, foods, medicines, drug delivery etc. Increased production and usage lead to augmented exposure of humans via oral intake and inhalation. Despite their widespread use, the potential health implications as well as molecular modes of action are not sufficiently known.

SiO₂-NPs enter cells and induce time- and size-dependent cytotoxicity at high concentrations by induction of oxidative stress [1], membrane damage, as well as disturbed calcium homeostasis [2,3]. In human intestinal Caco-2 cells, SiO₂-NPs induced genotoxicity and pro-inflammatory reactions [4]. In human lung cells, SiO₂-

NPs induce oxidative stress and inflammation [5]. The induction of pro-inflammatory responses after exposure to SiO₂-NPs was also found in human lung epithelial cells, human macrophages [6] and in the rat lung [7]. Recently, we have shown that SiO₂-NPs induce endoplasmic reticulum (ER) stress in human hepatoma cells [8], pro-inflammatory cytokine TNF- α and activate MAPK pathways [9].

The ER stress response is activated when folding and export of proteins is perturbed under different cellular stress conditions. Accumulation of non- or misfolded proteins in the ER induces an adaptive response, the unfolded protein response (UPR), that reduces protein de novo synthesis and enhances the production of chaperones that facilitate protein folding and enhances the proteosomal degradation of misfolded proteins [10]. There are three distinct pathways triggered by ER stress namely protein kinase like ER kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6), all of which induce the expression of different genes aiming to restore the normal function of the ER, and in case it fails, to induce apoptosis [11]. All three pathways are controlled through the chaperone binding immunoglobulin protein (BIP) (or also called 78 kDa glucose-regulated protein, GRP78).

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The ER stress response can be assessed by selective markers, such as induction of the chaperone BIP, splicing of XBP-1 mRNA, phosphorylation of eIF2 α and induction of CHOP. The UPR has important consequences, including apoptosis. It accompanies acute and chronic liver diseases and plays a significant role in liver pathogenesis [12]. Additionally ER stress can activate NF κ B [13] leading to the expression of interferons (INFs) Type I and inflammatory cytokines like TNF- α [14], which is involved in the inflammatory response, apoptosis, cell proliferation and cell differentiation.

Inflammatory and immune responses are regulated by multiple signalling pathways. Among them are the NF κ B and mitogen-activated protein kinase (MAPK) signalling pathways, which include many proteins including MAPK (originally called the extracellular signal-regulated kinase1/2 ERK1/2), p38, CREB, CMYC and CJUN N-terminal kinase (JNK) pathways. MAPK regulates the activities of many transcription factors. It is important in the cellular stress response, modulates a variety of inflammatory responses [15] and apoptosis, and plays a role in cancer development.

Based on our previous findings that SiO₂-NPs induced the expression of ER stress markers [8,9], and that SiO₂-NPs induced TNF- α expression and activated the MAP kinases regulated transcription factors CJUN and CMYC [9], here we aimed to further explore and deepen our understanding on ER stress, on induction of inflammatory response, and its consequences such as MAPK activation following exposure to SiO₂-NPs. To this end, we exposed human hepatoma cells Huh7 to SiO₂-NPs followed by a detailed investigation of ER stress response. Exposure concentrations were selected based on the previous study on cytotoxicity of SiO₂-NPs [8]. The used concentrations were non-toxic, therefore all detected effects are real effects and not unspecific toxic reactions. We analysed the expression of the chaperone *DNAJB9*, the ATF-4 target gene *GADD34* and the pro-apoptotic gene *CHOP*, the expression of *Il-8* as a marker for inflammation, as well as the expression of various genes belonging to the MAPK signalling pathway. Our study shows that all of these pathways are affected by exposure to SiO₂-NPs, suggesting that further toxicological assessment of human exposure is needed for safety assessment of these nanoparticles.

2. Materials and methods

2.1. Chemicals

SiO₂-NPs, saquinone and SP600125 were purchased from Sigma-Aldrich (Buchs, Switzerland), anti-CHOP antibody was purchased from BioConcept (Allschwil, Switzerland), and anti-Actin antibody was purchased from Fisher Scientific (Reinach, Switzerland), the secondary anti-mouse antibody IRDye 680RD was purchased from Lucerna-Chem (Lucerne, Switzerland).

2.2. Huh7 cells

The human hepatoma cell line Huh7 was kindly provided by Markus Heim, University Hospital Basel, Switzerland. Cells were grown in DMEM with GlutaMAX™ (LuBioScience, Lucerne, Switzerland) supplemented with 10% FCS for cultivation and without FCS for exposure experiments in a humidified incubator with 5% CO₂ at 37 °C. Cells were usually split every 4 days and sub-cultured at split ratios of about 1:6.

2.3. Characterization of the SiO₂-NPs

Surface characteristics, size distribution and appearance were previously reported [8].

2.4. RNA isolation, reverse transcription, and quantitative (q)PCR

Total RNA was isolated from Huh7 cells using Trizol reagent according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche, Rotkreuz, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. qPCR was performed based on SYBR green fluorescence (SYBR green PCR master mix; Roche, Rotkreuz, Switzerland). The sequences of the used primers are shown in Table 1. The amplification conditions were 95 °C for 5 min. For initial denaturing, 40 cycles of 95 °C for 30 s for denaturing, 61 °C for 60 s for annealing and elongation. A melting curve was run afterwards. The difference in the cycle threshold (Δ CT) value was derived by subtracting the CT value for GAPDH, which served as an internal control, from the CT value for the target genes. All reactions were run in duplicates using a BioRad real time PCR machine (CFX 96 Real Time System). mRNA expression levels of target genes were expressed as a several fold increase according to the formula $2^{\Delta\Delta CT}$ (not exposed)– Δ CT (exposed) in which “not exposed” are solvent control samples. Due to graphical reasons the fold change is converted into Log₂ values.

2.5. RT² profiler™ PCR array human MAP kinase signalling pathway

Sample preparations and the experimental procedures were performed according to manufacturer's instructions. Total RNA was isolated from Huh7 cells using the RNeasy kit (QIAGEN, Basel, Switzerland). cDNA was obtained using the RT² first strand kit (QIAGEN, Basel, Switzerland) using 500 ng RNA as input for both the control and the treated sample. The qPCR performed was based on SYBR green fluorescence and the provided RT² SYBR Green master mix was used. The amplification conditions were as described above. The obtained data were analysed using the QIAGEN web portal.

2.6. Preparation of cell extracts and immunoblotting

Cells were homogenized in 50 μ l of lysis buffer (50 mM Tris, 150 mM NaCl, 15 mM EDTA, 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) incubated for 20 min on ice, centrifuged at 14,000 rpm for 5 min. Protein concentrations were determined with Thermo Scientific BCA™ protein assay kit (Fisher Scientific, Wohlen, Switzerland). Immunoblotting was performed as described [16]. Membranes were incubated with the primary antibodies anti-CHOP and anti-actin and with the secondary fluorescent anti-mouse antibody. To detect the CHOP and actin band, the membranes were scanned with Licor Odyssey scanner (Bad Homburg, Germany). Analysis to determine the intensity of the CHOP and actin bands was performed with Image J software.

2.7. Measurement of TNF- α protein

The amount of TNF- α protein in the cell culture supernatant was measured using the Cymax human TNF- α ELISA kit from Adipogen (Liestal, Switzerland) according to the manufacturer's protocol.

2.8. Statistical analysis

Differences between treatments were assessed by one way ANOVA followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. Results are

Table 1
Sequences of used qPCR primers.

Primer	Forward (5' → 3')	Reverse (5' → 3')
<i>GAPDH</i>	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTTC
<i>ATF4</i>	AGT GGC ATC TGT ATG AGC CCA	GCT CCT ATT TGG AGA GCC CCT
<i>BIP</i>	CGA GGA GGA GGA CAA GAA GG	GAC CTT GAA CGG CAA GAA CT
<i>XBP-1s</i>	TGC TGA GTC CGC AGC AGG TG	GCT GGC AGG CTC TGG GGA AG
<i>NOXA</i>	ATT ACC GCT GGC CTA CTG TG	GTG CTG AGT TGG CAC TGA AA
<i>CHAC-1</i>	CCT GAA GTA CCT GAA TGT GCG AGA	GCA GCA AGT ATT CAA GGT TGT GGC
<i>TNF-α</i>	CAG CCT CTT CTC CTT CCT GA	TGAGGTACAGACCCCTCTGAT
<i>FRA-1</i>	CAG GCG GAG ACT GAC AAA CTG	TCC TTC CGG GAT TTT GCA GAT
<i>CHOP</i>	GGA GCA TCA GTC CCC CAC TT	TGT GGG ATT GAG GGT CAC ATC
<i>CFOS</i>	CCG GGG ATG CCT CTC TTA CT	CCAGGTCCCGTGCAGAAGTC
<i>PUMA</i>	CGA CCT CAA CGC ACA GTA CG	AGG CAC CTA ATT GGG CTC CAT
<i>CJUN</i>	TCC AAG TGC CGA AAA AGG AAG	CGA GTT CTG AGC TTT CAA GGT
<i>BIM</i>	GTA TTC GGT TCG CTG CGT TC	CGC AGG CTG CAA TGG TCT AC
<i>CMYC</i>	TGA GGA GAC ACC GCC CAC	CAA CAT CGA TTT CTT CCT CAT CTT
<i>GADD34</i>	CCC AGA AAC CCC TAC TCA TGA TC	GCC CAG ACA GCC AGG AAA T
<i>DNAJB9</i>	TCG GCA TCA GAG CGC CAA ATC A	ACC ACT AGT AAA AGC ACT GTG TCC AAG
<i>JUND</i>	TCA TCA TCC AGT CCA ACG GG	TTC TGC TTG TGT AAA TCC TCC AG

given as means ± standard error of means. Differences were considered statistically significant with one asterisk at $p = 0.01$ – 0.05 , two asterisks at $p = 0.001$ – 0.01 and three asterisks at $p = 0.0001$ – 0.001 .

3. Results

3.1. Characterisation of SiO₂-NPs

We characterized the used SiO₂-NPs before [8]. They had a surface area/weight ratio of 115 m² g⁻¹, and size of 273 nm. Their surface was negatively charged (−12.7 mV). Nanoparticle tracking analysis showed that 50% of the SiO₂-NPs had a size smaller than 100 nm, 40% a size between 100 and 200 nm, and 10% of the particles had sizes larger than 200 nm. As most of the particles showed a size smaller than 100 nm, potential biological activity can be expected.

3.2. Induction of ER stress

Exposure of Huh7 cells to SiO₂-NP induced ER stress, as demonstrated by dose-related transcriptional induction of ER stress genes, *DNAJB9*, *GADD34* and *CHOP* upon exposure to 10 and 50 μg/ml SiO₂-NPs after 24 h (Fig. 1A). Additionally, CHOP protein was induced after 24 h exposure to 50 μg/ml SiO₂-NPs (Fig. 1B).

As CHOP plays an important role in the induction of apoptosis under prolonged ER stress, we further investigated the expression of CHOP target genes. The transcripts of *BIM*, *CHAC-1*, *NOXA* and *PUMA* were significantly induced upon 24 h exposure to 50 μg/ml SiO₂-NPs (Fig. 1C). In addition to ER stress, SiO₂-NPs induced an inflammatory response by induction of TNF-α and *IL-8*; there was a dose-related induction of the *IL-8* transcript after 24 h exposure to 10 and 50 μg/ml SiO₂-NPs (Fig. 1D).

3.3. Activation of MAPK signalling

To investigate molecular effects of SiO₂-NPs on the activation of MAP kinases, a MAPK signalling pathway PCR array was performed. Table 2 shows that five of the totally 84 analysed genes exhibited a significant transcriptional up-regulation, and 18 genes a significant down-regulation upon exposure of Huh7 cells to SiO₂-NPs. Of the five up-regulated genes, *P21* and *P19* encode for inhibitors for the cyclin D and the CDK4/6 complex; *CFOS* and *CJUN* are downstream of ERK and JNK kinases, and *KSR1* promotes MEK and RAF phosphorylation. Of the 18 down-regulated genes, strongest down-regulation occurred for the proto-oncogene *BRAF*, *MAPK11*, one of the four p38 MAPK genes, and for *NFATC4*, which is important for T-cell development (Fig. 2A).

To verify the results of the PCR array, transcriptional expression of *CFOS* and *CJUN* was assessed after SiO₂-NP exposure. There was a strong induction of both genes after exposure to 50 μg/ml SiO₂-NPs for 24 h (Fig. 2B). In addition, the expression of another downstream target of MAPKs was assessed. *FRA1* showed an up-regulation upon SiO₂-NP exposure (Fig. 2B). Compared to the other three analysed MAPK targets, *CMYC* showed a significant but weaker up-regulation (Fig. 2B).

To analyse for the effects driven by MAPK activation, Huh7 cells were co-exposed to 50 μg/ml SiO₂-NPs and to 25 μM of the JNK inhibitor SP600125 for 24 h. There was a significant down-regulation of the *JUND*, *CJUN*, *CFOS* and *FRA1* transcripts. In contrast, *CMYC* transcript showed a significant up-regulation (Fig. 2C). The partial inhibition of the MAPK signalling had no effect on the expression of the *TNF-α* transcript (Fig. 2D). There seems to be no direct link between MAPKs and ER stress. The up-regulation of *BIP*, *XBP1s*, *DNAJB9*, *CHOP* and *GADD34* transcripts in response to SiO₂-NPs was not significantly changed, when partially inhibiting the MAPK signalling (Fig. 2E).

3.4. Effects driven by SiO₂-NPs induced TNF-α

To analyse for the effects derived from up-regulation of TNF-α, Huh7 cells were exposed to SiO₂-NPs in the presence of the TNF-α inhibitor sauchinone. Sauchinone reduced the induction of the TNF-α transcript upon SiO₂-NP exposure by about 50% (Fig. 3A). On protein level, however, no TNF-α was detectable after co-exposure of Huh7 cells to SiO₂-NPs and sauchinone (Fig. 3B). Furthermore, to address the question, whether SiO₂-NPs induced ER stress, and in turn TNF-α, or vice versa, the expression of ER stress genes upon co-exposure of Huh7 cells to SiO₂-NPs and sauchinone was assessed. The reduction in the TNF-α level lead to a significant reduction of transcripts of ER stress genes *BIP*, *XBP-1s* and *DNAJB9* (Fig. 3C). Furthermore, transcripts of *ATF-4*, *GADD34* and *CHOP* were lowered in the presence of sauchinone (Fig. 3C).

The effects of TNF-α on MAPK expression were investigated by analysing the expression of *FRA1*, *CFOS*, *CMYC* and *CJUN*. The transcript of *CFOS* was significantly reduced in the presence of sauchinone, the other three analysed transcripts did not show any significant changes (Fig. 3D).

4. Discussion

4.1. Induction of ER stress response

In the present study, we analysed in detail the induction of ER stress and evaluated the consequences of the induction of TNF-α

Table 2

Alteration of expressed genes from the MAPK array. Significantly up-regulated genes are shown in red, significantly down-regulated genes in green.

Symbol	Fold Change	Log2
BRAF	0.23	-2.15
CCND1	0.41	-1.3
CCND3	0.51	-0.98
CDK2	0.54	-0.89
CDK6	0.54	-0.89
CDKN1A	5.56	2.48
CDKN1C	0.54	-0.9
CDKN2B	0.68	-0.55
CDKN2C	0.39	-1.37
CDKN2D	5.9	2.56
CHUK	0.58	-0.79
CREBBP	0.51	-0.98
DLK1	0.39	-1.35
EGFR	0.35	-1.52
EGR1	1.56	0.64
FOS	12.08	3.59
GRB2	0.59	-0.75
HSPB1	0.59	-0.76
JUN	8.49	3.09
KRAS	0.42	-1.25
KSR1	2.17	1.12
LAMTOR3	1.54	0.63
MAP2K2	0.7	-0.52
MAP2K3	1.63	0.71
MAP2K4	0.61	-0.71
MAP2K5	0.36	-1.49

Table 2 (Continued)

MAP2K6	0.48	-1.06
MAP3K2	0.51	-0.96
MAP3K3	0.62	-0.68
MAP3K4	0.44	-1.19
MAPK1	0.55	-0.87
MAPK11	0.24	-2.04
MAPK12	0.59	-0.76
MAPK14	0.41	-1.27
MAPK3	0.36	-1.49
MAPK7	0.47	-1.08
MAPK9	0.49	-1.02
MAPKAPK2	0.54	-0.89
MKNK1	0.63	-0.66
MST1	0.47	-1.08
MYC	1.84	0.88
NFATC4	0.29	-1.78
NRAS	0.6	-0.73
PRDX6	0.69	-0.53
RAF1	0.51	-0.96
RB1	0.48	-1.06
SFN	0.46	-1.11

and activation of MAPK signalling pathway by SiO₂-NPs at exposure concentrations of 10 and 50 µg/ml. Based on a Dutch food consumption survey [17], the daily up-take of amorphous SiO₂-NPs as food additives was estimated to be 9.4 mg per kg body weight [18]. This would make a daily uptake of 658 mg SiO₂-NPs for a 70 kg weight man. *In vitro* systems mimicking human gastric digestion showed that 80% of orally ingested SiO₂-NPs withstand gastric dissolution and display still nano-size range [19]. Based on these data, our effects at 10 and 50 µg/ml SiO₂-NPs are relevant regarding human health issues. In previous studies, we demonstrated the induction of various ER stress markers in Huh7 cells upon exposure to SiO₂-NPs [8,9].

The induction of ER stress is also known for other nanoparticles. Titanium dioxide nanoparticles induced ER stress and inflamma-

tion in the lung of mice after inhalation [20]. Silver nanoparticles induced ER stress in different cell lines and in lung, liver and kidneys of mice after intratracheal instillation [21]. Zinc oxide nanoparticles induced ER stress in liver tissue of mice [22]. The induction of ER stress by zinc oxide nanoparticles was also observed *in vitro* and can be regarded as an early biomarker for nanoparticle toxicity [23]. These findings suggest that the induction of ER stress is a mode of action of different nanoparticles.

In addition to the common ER stress markers BIP and XBP-1s, here we also analysed the abundance of the *DNAJB9* and *GADD34* transcripts. *DNAJB9* is a chaperone that is involved in the ER-associated degradation (ERAD) of misfolded proteins. *GADD34* plays a role in dephosphorylation of the translation initiation factor eIF-2α and is a promoter of apoptosis and *CHOP*, a gene which plays

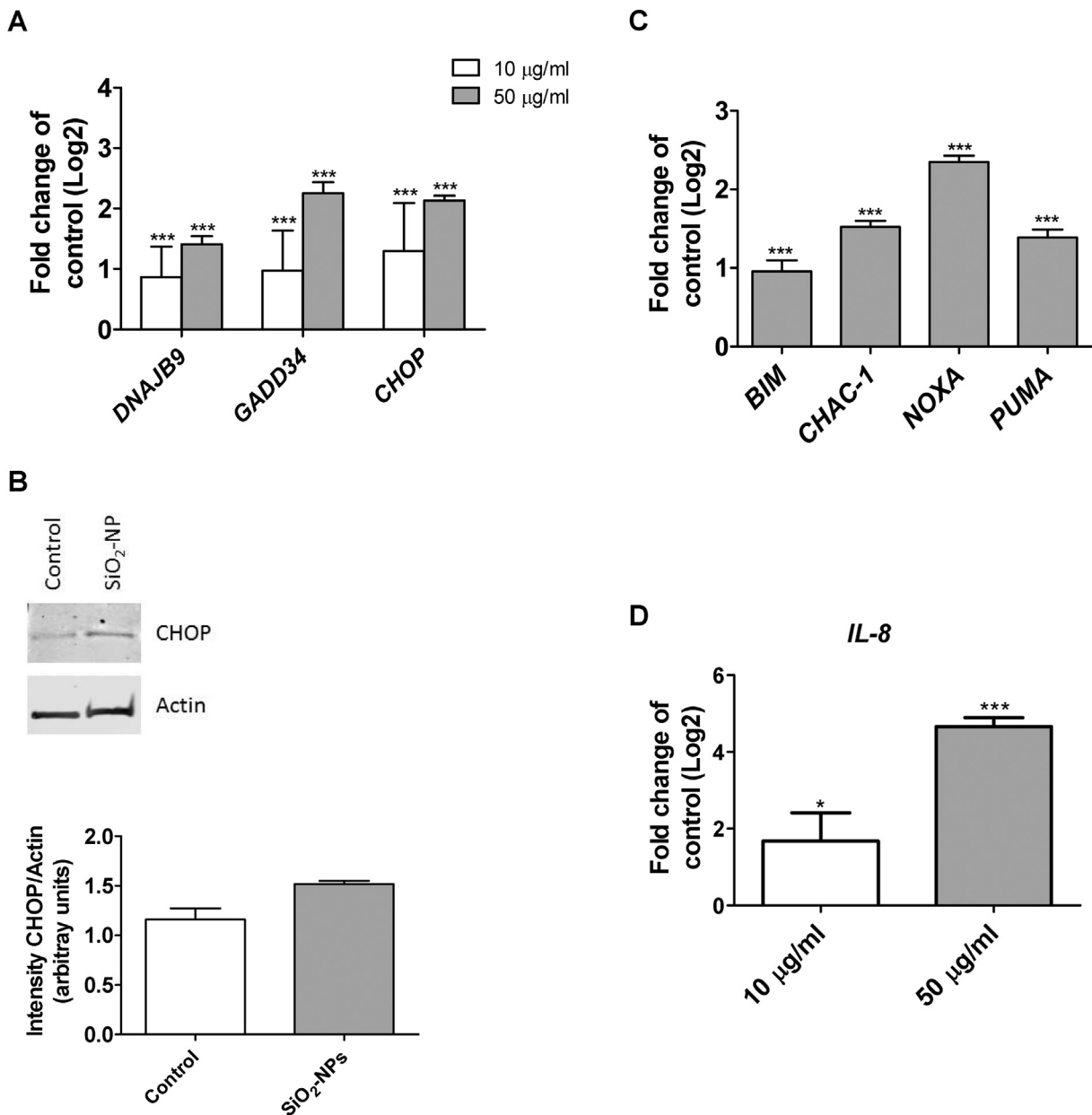


Fig. 1. Induction of ER stress and inflammation in response to SiO₂-NP exposure of Huh7 cells. **A:** Abundance of *DNAJB9*, *GADD34* and *CHOP* transcripts following exposure to 10 (white bars) and 50 $\mu\text{g/ml}$ (grey bars) SiO₂-NPs for 24 h. **B:** Huh7 cells were exposed to 50 $\mu\text{g/ml}$ SiO₂-NPs for 24 h followed by Western Blot analysis for CHOP and actin (above), and analysis of the Western Blot signal intensities (below). **C:** Expression of CHOP target genes *BIM*, *CHAC-1*, *NOXA* and *PUMA* in Huh7 cells following exposure to 50 $\mu\text{g/ml}$ SiO₂-NPs for 24 h. **D:** Abundance of *IL-8* transcript in Huh7 cells following exposure to 50 $\mu\text{g/ml}$ SiO₂-NPs for 24 h. Shown are results of three independent experiments \pm SEM. Significant differences are marked with one asterisk at $p=0.01$ – 0.05 , two asterisks at $p=0.001$ – 0.01 and three asterisks at $p=0.0001$ – 0.001 .

a role in apoptosis. Exposure of Huh7 cells to SiO₂-NP resulted in a strong induction of *GADD34* and *CHOP*, and a weaker but significant induction of *DNAJB9* (Fig. 1A). *GADD34* is a very interesting protein as it plays a role in the induction or promotion of various cancers, including colon cancer in mice by induction of pro inflammatory mediators like TNF- α , and by induction of epithelial proliferation [24]. Up-regulation of *GADD34* was noted in the liver of mice exposed to diethylnitrosamine. It induced the up-regulation of pro inflammatory cytokines and expression of the oncogene C-MYC leading to the development of hepatocellular cancer [25].

In general, it is still unclear, whether ER stress induces TNF- α expression, or whether TNF- α induces ER stress. In mouse embryonic fibroblasts, ER stress induced TNF- α [26]. In another study, ER stress induced IRE1 α activation regulated the production of inflammatory cytokines, such as TNF- α [27]. Exposure of bone marrow

stem cells to alcohol induced up-regulation of *BIP*, *ATF-4*, *CHOP* and *TNF- α* . Simultaneous silencing of *ATF-4* and *CHOP* prevented the induction of TNF- α [28]. On the other hand, induction of the unfolded protein response upon TNF- α exposure was shown in murine fibrosarcoma cells [29].

The induction of a pro-inflammatory response by SiO₂-NPs was shown in human lung cells and murine macrophages, where a strong transcriptional induction of the pro-inflammatory cytokines TNF- α , *IL-6* and *IL-8* was noted [6]. Another link between ER stress and induction of inflammatory cytokines is the activation of intracellular NOD receptors, normally activated by binding to bacterial structures, through ER stress, followed by induction of inflammatory cytokines. The increased activity of the enzyme IRE1 α in response to ER stress, leads to the formation of a complex between

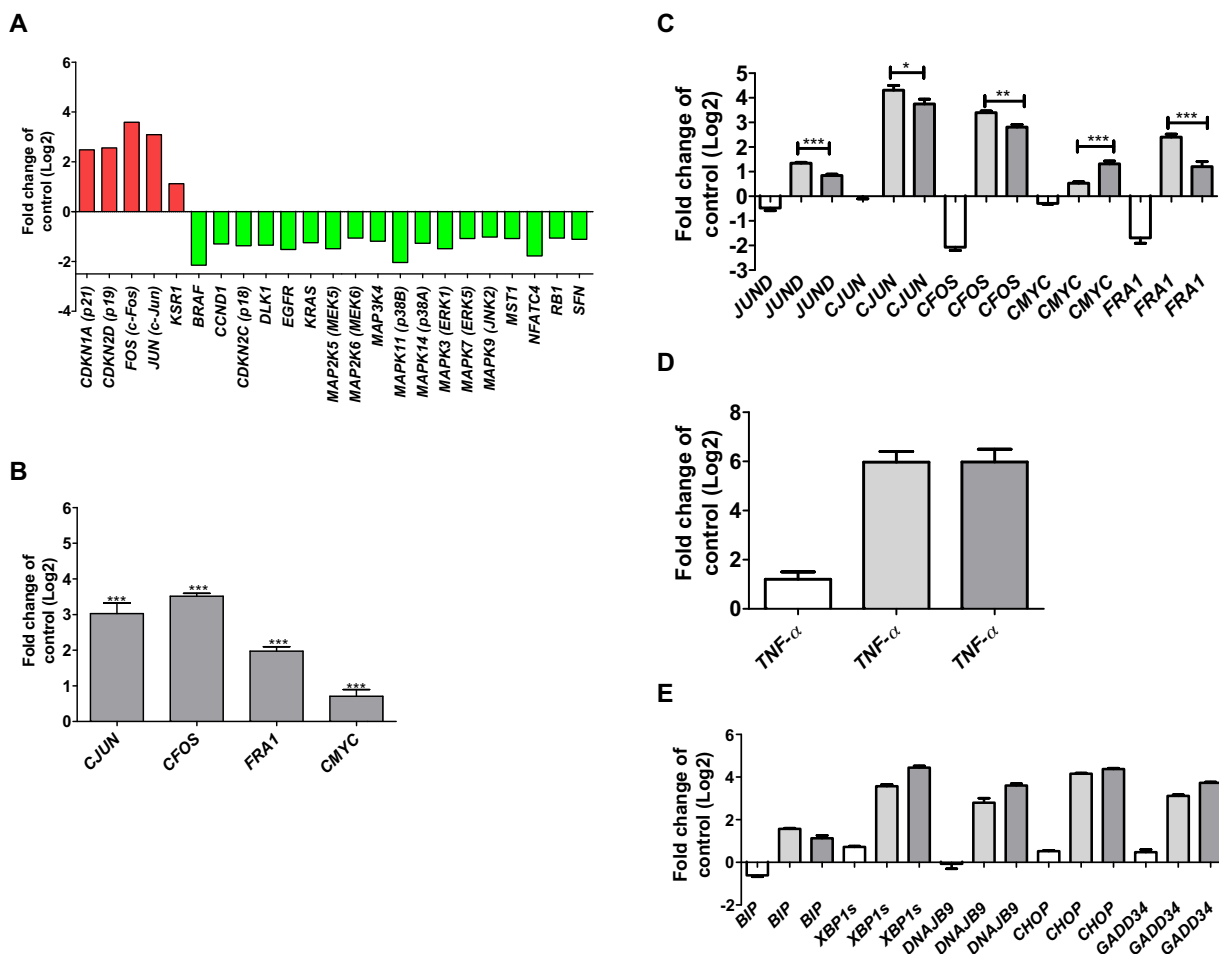


Fig. 2. Activation of MAPK signalling in Huh7 cells in response to SiO₂-NPs. A: Transcripts from the human MAP Kinase Signaling Pathway RT² ProfilerTM PCR Array showing significant up- and down-regulation in Huh7 cells following exposure to 50 μg/ml SiO₂-NPs after 24 h. B: Abundance of *CJUN*, *CFOS*, *FRA1* and *CMYC* transcripts in Huh7 cells following exposure to 50 μg/ml SiO₂-NPs for 24 h. C: Abundance of *JUND*, *CJUN*, *CFOS*, *FRA1* and *CMYC* transcripts in Huh7 cells following co-exposure to 50 μg/ml SiO₂-NPs and 25 μM JNK inhibitor SP600125 for 24 h. Key for C, D and E: white bars: 25 μM SP600; light grey bars: 50 μg/ml SiO₂-NPs; dark grey bars: 25 μM SP600 and 50 μg/ml SiO₂-NPs. Shown are results of three independent experiments +/-SEM. Significant differences are marked with one asterisk at p=0.01–0.05, two asterisks at p=0.001–0.01 and three asterisks at p=0.0001–0.001.

the TRAF2, NOD1 and NOD2, initiating inflammation through RIP2 activation [30].

In our study, we aimed to clarify this question by exposure of Huh7 cells to SiO₂-NPs and the TNF-α inhibitor sauchinone. Indeed, sauchinone reduced the up-regulation of *TNF-α* mRNA upon SiO₂-NP exposure about 50% (Fig. 3A) and eliminated the TNF-α protein (Fig. 3B). As a consequence, the induction of the ER stress marker gene transcripts were significantly reduced (Fig. 3B). This leads to the conclusion that SiO₂-NPs first led to induction of TNF-α, and subsequently to induction of ER stress.

4.2. Activation of MAPK signalling

To investigate the effect of SiO₂-NPs on the MAPK signalling pathway, the expression of 84 different MAPK signalling related genes was assessed. Strong up-regulation was found for *CDKN1A/P21*, *CDKN2D/P19*, *CFOS* and *CJUN*, and a weaker up-regulation of *KSR1* (Fig. 2A). This strong induction of MAPK target genes gives a strong hint that the MAP kinases are activated upon SiO₂-NP exposure even if the phosphorylation of the MAP kinases was not assessed. *CDKN1A/P21* is a tumor suppressor gene involved in the regulation of cell cycle progression in the G1 phase. It also plays a crucial role in the development of hepatocellular cancer.

Hepatitis B virus X protein (HBx) induces hepatocarcinogenesis by induction of *P21* overexpression [31].

CJUN plays a role in several cellular processes including proliferation, cell survival and cell transformation [32,33]. In human hepatocellular cancer *CJUN* is activated suggesting an important oncogenic function [34]. *CJUN* prevents apoptosis by antagonizing p53 activity, and thus contributes to the early stage of human hepatocellular cancer development [35], or liver tumor initiation [36]. In human liver biopsies from hepatocellular carcinoma patients infected with hepatitis C virus, protein kinase R lead to an overexpression of *CFOS* and *CJUN* promoting proliferation of hepatocellular carcinoma [37].

In our study, SiO₂-NPs led to a strong induction of *CJUN* transcript (Figs. 2A, B). In the presence of the JNK inhibitor SP600125, induction was significantly repressed. Furthermore, the expression of *CFOS* and *FRA1* was significantly repressed by SP600125 (Fig. 2C). The inhibition of *JNK* had no effects on the abundance of the *TNF-α* transcript and on the activation of ER stress (Figs. 2D and E). This leads to the conclusion that these two events are up-stream of the MAPK activation upon SiO₂-NP exposure.

The inhibition of TNF-α by sauchinone had a weaker effect on the activation of MAPKs. Only *CFOS* was significantly reduced (Fig. 3D). We hypothesize that the induction of ER stress is responsible for the activation of the MAPKs. To further clarify this point, exper-

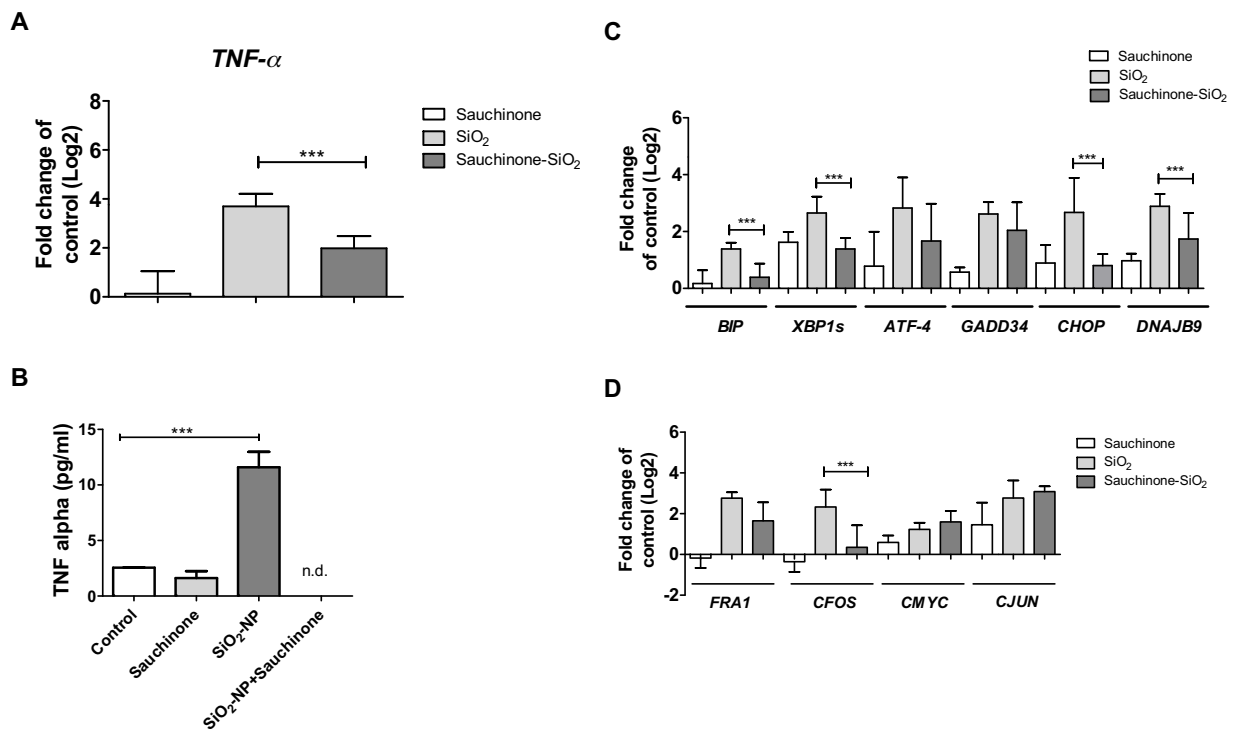


Fig. 3. Alterations in gene expression driven by *TNF- α* . Abundance of *TNF- α* transcript (A), and *TNF- α* protein (B), of *BIP*, *XBP1s*, *ATF-4*, *GADD34*, *CHOP* and *DNAJB9* transcripts (C), and *FRA1*, *CFOS*, *CMYC* and *CJUN* transcripts (D) in Huh7 cells following co-exposure to 50 $\mu\text{g/ml}$ SiO_2 -NPs and 25 μM sauchinone for 24 h. Shown are results of three independent experiments \pm SEM. Significant differences are marked with one asterisk at $p=0.01$ – 0.05 , two asterisks at $p=0.001$ – 0.01 and three asterisks at $p=0.0001$ – 0.001 .

iments with *TNF- α* blocking antibodies should be done in future experiments. It is known that ER stress can activate MAPKs, and as a consequence, induce the activation of the AP-1 complex [38]. Consequently, ER stress may be involved in chronic liver diseases.

SiO_2 -NPs were shown in different cells to induce reactive oxygen species (ROS) and oxidative stress. We previously demonstrated this reaction in Huh7 cells in response to SiO_2 -NPs coated with 1% silver [8]. Induction of ROS and oxidative stress was also found in kidney cells [1], human lung cells [5], murine lung epithelial cells [39], and murine macrophages [40]. As oxidative stress can lead to the induction of ER stress [41], induction of oxidative stress by SiO_2 -NPs could be an additional mechanisms responsible for the activation of ER stress.

5. Conclusions

Our data show a clear induction of an inflammatory response by *IL-8* and *TNF- α* induction upon SiO_2 -NP exposure. One of the consequences of elevated *TNF- α* levels is the induction of ER stress, which in turn can activate the MAPKs leading to the activation of the AP-1 complex. More research is needed to analyse the link between SiO_2 -NP exposure, induction of chronic inflammation, induction of ER stress, and induction of MAPK in relation to health impacts.

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