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Author agreement

We declare that the manuscript entitled "4-Nonylphenol reduces cell viability and induces apoptosis and ER-stress in a human epithelial intestinal cell line" by Lepretti et al. for submission in *Experimental and Toxicologic Pathology* contains findings that have not been published previously, nor are under consideration by any other journal.

We declare no conflict of interest.

We declare that all of us have substantially contributed to the article, as here specified: conceived and designed the study, IC, AM, FG, AC; performed the experiments, collected, analyzed and interpreted the data, ML, GP, DG, IC, AM; drafted and revised the manuscript, IC, AM, ML, AC, FG, CE; contributed for reagents and materials, IC, AM, CE.

We have read and approved the version of the manuscript being submitted.

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***Cover Letter**

Dear Editor

On behalf of my co-authors, I am hereby submitting an original article entitled "4-Nonylphenol reduces cell viability and induces apoptosis and ER-stress in a human epithelial intestinal cell line" by Lepretti et al. for publication in *Experimental and Toxicologic Pathology*.

In this study we investigated the effects of 4-nonylphenol, an ubiquitous environmental pollutant contaminating food and water, in a cell line of human intestinal epithelium. Many studies have been conducted in several cell and tissue models demostrating toxic properties of 4-nonylphenol. However, little is known about harmful effects of this compound at the intestinal level, even if intestine may be one of the first organs to be damaged by chronic exposure to 4-nonylphenol through the diet. Here we demonstrated that 4-nonylphenol was cytotoxic for intestinal cells, as it reduces cell surviving ability and triggers stress response and death programs. Our findings suggest that exposure to 4-nonylphenol through the diet may lead to local intestinal damage and negatively affect intestinal homeostasis.

We believe that the data reported here will be of interest to the wide readership of *Experimental and Toxicologic Pathology*.

We look forward to hearing from you,

Yours sincerely,

Ivana Caputo

4-Nonylphenol reduces cell viability and induces apoptosis and ER-stress in a human epithelial intestinal cell line

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Abstract

4-Nonylphenol is a widely diffused and stable environmental contaminant, originating from the degradation of alkyl phenol ethoxylates, common surfactants employed in several industrial applications. Due to its hydrophobic feature, 4-nonylphenol can easily accumulate into living organisms, including humans, where it displays a wide range of toxic effects. Since the gastrointestinal tract represents the main route by which 4-nonylphenol enters into the body, intestine may be one of the first organs to be damaged by chronic exposure to this pollutant through the diet. With the present study, we aimed to investigate the effects of 4-nonylphenol on a human intestinal epithelial cell line (Caco-2 cells). We demonstrated that 4-nonylphenol was cytotoxic to cells, as revealed by a decrease of the cell number and the decrement of mitochondrial functionality after 24 h of treatment. 4-Nonylphenol also reduced the number of cells entering into S-phase and interfered with epidermal growth factor-signalling, with consequent negative effects on cell ability to survive. In addition, 4-nonylphenol induced apoptosis, involving the activation of caspase-3, and triggered an endoplasmic reticulum-stress response, as resulted from 78 kDa glucose-regulated protein over-expression and X-box binding protein-1 activation. Altogether, these findings support the hypothesis that prolonged exposure to 4-nonylphenol through the diet may lead to a local damage at the level of intestinal mucosa, with potential negative consequences for intestinal homeostasis and functionality.

Key words: 4-nonylphenol; Caco-2 cells; apoptosis; cell proliferation; endoplasmic-reticulum stress; intestinal toxicity.

Introduction

Alkyl phenol ethoxylates are compounds widely used as non-ionic surfactants in several industrial applications. They are components of industrial or domestic cleaning products, present in shampoos and cosmetics, used as antioxidant for plastics and rubbers, and as antifogging in food-packaging polymers (Lorenc et al., 1992). They are also employed as additives in pesticides and lubricant oil. For their uses, alkyl phenol ethoxylates are commonly found in waste waters and sediments, where they are transformed by environmental microorganisms to more stable compounds, such as 4-nonylphenol (4-NP)[1] and short-chain NP ethoxylates (Soares et al., 2008). Due to its hydrophobic feature, 4-NP can easily be absorbed by living organisms, especially by water species, and accumulates mainly into adipose tissue, but also into some organs, where it can reach concentrations up to 1000-fold higher than those found in the environment. Several studies have reported toxic effects of 4-NP in invertebrates and in vertebrates (Hong and Lin, 2007; Jie et al., 2013; Lagadic et al., 2007). Since 4-NP structure mimics estrogens, it has a relatively high affinity for estrogens receptors (Kuiper et al., 1998). As a consequence, 4-NP can induce estrogenic responses (Teresaka e al., 2006) and it has been recognized as a common endocrine-disrupter (Shanle and Xu, 2011), thus causing alteration of the reproductive biology and development abnormalities in amphibians and fishes (Capaldo et al., 2012; Mosconi et al., 2002; Popek et al., 2006), as well as in mammals (Laws et al., 2000). In addition, a direct cytotoxicity of 4-NP has been observed, as it can affect survival and induce apoptosis in several cell and animal models (Jubendradass et al., 2012; Manente et al., 2011; Mao et al., 2008). 4-NP environmental contamination also causes human exposure, that may occur by dermal absorption, inhalation and overall ingestion of contaminated foods (mainly fish and vegetables) and water. Increasing evidences indicate that commonly consumed food, including those for childhood, contain high 4-NP concentrations (Ferrara et al., 2005; Guenther et al., 2002; Raecker et al., 2011). Consequently, dietary daily intake of 4-NP for adults has been estimated to be 7.5 μg in a German study (Guenther et al., 2002) and even 30 μg in a Chinese study (Lu et al., 2007). 4-NP has also been detected in human breast milk, giving a mean daily intake of 4-NP up to 2.2 μg/Kg of body weight (Ademollo et al., 2008). Moreover, 4-NP has been shown to transfer across human placenta in an *ex-vivo* model of placental perfusion (Balakrishnan et al., 2011). Up to date, many studies have been conducted to investigate 4-NP toxicity on cell and tissue models which are relevant for human health (Bennasroune et al., 2012; Suen et al., 2012; Wada et al., 2007). However, the molecular mechanisms of 4-NP multiple effects have not been fully explored. Since gastrointestinal tract represents the main route of 4-NP uptake for humans, intestine may be one of the first organs to be damaged by 4-NP chronic exposure through the diet. With the present study, we investigated the

molecular mechanisms underlying the potential 4-NP toxicity for human intestine. To this aim, we monitored cell viability and proliferating ability of a human intestinal epithelial cell line in the presence of increasing concentrations of 4-NP. We also evaluated whether 4-NP was able to trigger an apoptotic response and a stress condition involving the endoplasmic reticulum (ER).

Methods

Cell culture and treatments

Caco-2 cells were obtained from Interlab Cell Line Collection (Centro di Biotecnologie Avanzate, Genoa, Italy). Caco-2 cells were cultured in 100x10 mm Petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen SRL, Milan, Italy). Cells were maintained at 37°C in a 5% CO_2, 95% air-humidified atmosphere and passaged twice a week. Stock solutions of 4-NP, etoposide (an apoptosis-inducer) and thapsigargin (THP) (an ER-stress inducer) were prepared in dimethylsulfoxide (DMSO) (all from Sigma-Aldrich, Milan, Italy). For each treatment, the final concentration of DMSO in the culture medium was 0.05%. For 4-NP, a dose-range of 1-100 µM was generally used. Etoposide and THP were used at concentration of 0.5 mM and 1 µM, respectively. The final amount of H_2O_2, used as positive control in viability assays, was 0.05%.

Cell viability assays

To assess cell viability in the presence of 4-NP, cells were seeded at the density of $5.0x10^4/cm^2$ and cultured for 48 h, then treated with 4-NP at different concentrations for 24 h. Cells were harvested with trypsin/EDTA (Invitrogen), washed with phosphate-buffered saline (PBS) and counted by using a Burker haemocytometer. Before counting, cells were stained with Trypan blue dye (Sigma-Aldrich) (diluted 1:1 with cell suspension) to detect the presence of dead cells. Cell viability was also determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The MTT assay is based on the enzymatic conversion of MTT in mitochondria (Mosmann, 1983). After incubation with 4-NP, 0.5 mg/mL MTT was added to cell medium and incubated for 3 h at 37°C to allow MTT to be metabolized. The resulting formazan crystals were dissolved in DMSO and absorbances were measured at 570 nm.

S-phase entry assay

The bromodeoxyuridine (BrdU) (Roche Diagnostics SpA, Monza, Italy) incorporation study was carried out as reported elsewhere (Caputo et al., 2010) with some modifications. Briefly, Caco-2

cells were seeded on cover slips and, 48 h later, were challenged with different concentrations of 4-NP for 18 h to analyse the effect of 4-NP on cell proliferation. In alternative, cells were starved by culturing in 0.1% serum-containing medium for 24 h, then were induced to proliferate by adding epidermal growth factor (EGF) 20 nM, in the absence or in the presence of different amounts of 4-NP for 18 h. In both cases, 3 h before paraformaldehyde fixing and Triton X-100 permeabilizing, BrdU was added to the medium (final concentration, 100 μM). BrdU incorporation was monitored by treating cells with an anti-BrdU antibody 1:100 (Invitrogen) and a secondary TRITC-conjugated antibody (Invitrogen) 1:100. Finally, cells were stained for 5 min with Hoechst (1 μg/mL final in PBS) (Sigma-Aldrich) and mounted with Mowiol. The number of cells that entered into S-phase was expressed as the ratio between the number of cells incorporating BrdU and the total number of cells. Stained cells were observed with an AxioSkop 40 fluorescent microscope (Carl Zeiss MicroImaging, Inc.). Images were acquired with Axiocam MRc5 and processed with the Axiovision 4.2 software (Carl Zeiss MicroImaging Inc.). Over 300 cells in several fields were evaluated in each sample.

Microscopic apoptosis detection

To detect cell apoptosis, cells were seeded on glass cover slips and after 48 h were treated with 4-NP 1 μM and 10 μM, or with etoposide, for further 24 h, then were stained with Hoechst as described for S-phase entry assay. The appearance of apoptotic condensed chromatin was detected by microscope observation as reported above.
In alternative, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay was performed. A commercial TUNEL assay kit (Fragment End Labelling (FragEL™) DNA Fragmentation Detection Kits, Calbiochem) was used according to the manufacturer-provided protocol. Microscope observations were carried out as reported above.

Caspase-3 assay

We detected the caspase-3 activity by a colorimetric assay based on the hydrolysis of the synthetic tetrapeptide, acetyl-Asp-Glu-Val-Asp, labelled with p-nitroanilide (Sigma-Aldrich). Briefly, cells were cultured for 24 h with different amounts of 4-NP or with etoposide. After treatment, cells were harvested in PBS and lysates were prepared as described in the manufacturer's technical bulletin. Protein content of each lysate was determined by using the Bio-Rad protein assay, then 30 μg of proteins were incubated for 2 h at 37°C in a reaction mixture containing the labelled substrate (50 μM). The release of p-nitroanilide was monitored by reading absorbance on a spectrophotometer at a wavelength of 405 nm.

X-box binding protein-1 (XBP1) splicing

Caco-2 cells were cultured for 48 h, then treated with different amounts of 4-NP, or alternatively with THP, for 60 min and 5 h. Total RNA was extracted with the Trizol-reagent (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA synthesis reaction was performed using the QuantiTech Reverse Transcription Kit (Qiagen) by using 1 μg of total RNA. The obtained cDNAs were used to detect the unspliced and the spliced form of XBP1 by PCR. Primers for human XBP1 were: upper, 5'-CCTGGTTGCTGAAGAGGAGG-3'; lower, 5'-CCATGGGGAGATGTTCTGGAG-3'. They were used at 500 nM each. PCR reactions were run on a MyCycler™ Thermal Cycler System (Bio-Rad) for 29 cycles with heating at 94°C for 30 sec, followed by annealing at 60°C for 30 sec and polymerization at 72°C for 60 sec. For amplification of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) we used the following primers: upper, 5′-TTCAACAGCGACACCCACTG-3′; lower, 5′-CACCCTGTTGCTGTAGCCA-3′. PCR reactions were performed for 26 cycles with heating at 94°C for 30 sec, followed by annealing at 65°C for 45 sec and polymerization at 72°C for 2 min. Reactions from XBP1 and GAPDH amplification were run on 2% and 1% agarose gels, respectively, and visualized by ethidium bromide staining under UV excitation.

Real-time RT-PCR for analysis of 78 kDa glucose-regulated protein (GRP78) expression

Caco-2 cells were cultured for 48 h, then treated with different amounts of 4-NP, or alternatively with THP, for 5 h. RNA extraction and cDNA synthesis were performed as described above. The obtained cDNAs were used to amplify human GRP78 transcripts with the following primers: upper, 5′-CTGGGTACATTTGATCTGACTGG-3′, lower, 5′-GCATCCTGGTGGCTTTCCAGCCATTC-3′. The concentration of mRNA was normalized to the concentration of the transcript for GAPDH, which was amplified with the same primers already used for conventional PCR. For amplification with the real-time PCR, cDNA samples were analyzed in triplicate with the iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy) and using the iQ^{TM} 5 Multicolor Real Time PCR Detection System (Bio-Rad Laboratories). PCR reactions were performed with 250 nM of each primer and 10 μl of SYBR Green Supermix, in a total volume of 20 μl. The PCR program started with 3 min of incubation at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 60°C, and 20 sec at 72°C.

Western blot analyses

For the detection of the phosphorylated form of extracellular signal-regulated kinase (p-ERK), cells were first cultured with 0.1% serum-containing medium for 24 h, then treated with 4-NP 25 μM for

25 min, and with EGF 20 nM for the following 5 min. At the end of the treatment, cell were washed with PBS and mechanically harvested in lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% sodium dodecyl sulphate, 1% Triton X-100, 1 mM ortovanadate, 2 mM PMSF, 10 mM NaF and inhibitors cocktail (Sigma-Aldrich). After 30 min of incubation on ice, cell extracts were centrifuged at 12,000 g for 30 min at 4°C, to remove cell debris, then 75 µg of total proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrotransferred to a PVDF membrane. The blots were treated with 5% skim milk in PBS for 30 min, then incubated overnight at 4°C with a mouse anti-p-ERK monoclonal antibody (clone E4, 1:1,000; Santa Cruz, CA, USA). After washing, the blots were incubated for 1 h at room temperature with an anti-mouse-peroxidase secondary antibody (1:10,000; Bio-Rad Laboratories). Total ERK was detected on same blots using a rabbit anti-ERK polyclonal antibody (clone K23, 1:1000; Santa Cruz) and a rabbit-peroxidase secondary antibody (1:10,000; Bio-Rad Laboratories). Immunocomplexes were revealed using a chemiluminescence detection kit (Euroclone) according to the manufacturer's instructions. To detect GRP78 protein expression, cells were treated for 24 or 72 h with different amounts of 4-NP, or with THP, then cell lysates were prepared and western blot analysis was performed as previously described (Caputo et al., 2012). GRP78 protein was detected by using a rabbit anti-GRP78 polyclonal antibody (H-129, 1:1000; Santa Cruz). For normalization, a mouse anti-GAPDH antibody (Santa Cruz) was used.

Statistics

All data were expressed as mean ± SD; statistical analysis was performed by using the Student's t test. In all experiments, differences were considered to be statistically significant at $p<0.05$.

Results

4-NP affected cell viability

We performed viability assays on Caco-2 cells to assess 4-NP toxicity to intestinal epithelium. We observed that 4-NP dose-dependently reduced the number of living cells in the range 10-100 µM, with about 60% of reduction at 100 µM (Figure 1A). Additionally, 4-NP caused a significant increase of cell necrosis, evaluated by counting cells stained by the Trypan blue dye; indeed, we found 5.3±1.1%, 11.6±7.4% and 19.6±5.0% of dead cells, with respect to total cells, after treatment with 10, 50 and 100 µM of 4-NP, respectively. The toxic effect of 4-NP was also confirmed by an MTT assay. We found that 4-NP induced a significant reduction of the mitochondrial activity already at concentration of 1 µM, while at concentration of 100 µM the reduction was more than

50% (Figure 1B). As expected, the cytotoxic agent H_2O_2, used as positive control, induced a decrease of cell viability over 90%.

4-NP interfered with proliferative signals

Since we observed a reduced number of viable cells after 4-NP treatment, we decided to investigate whether 4-NP could also affect cell cycle progression and interfere with proliferative signals. By performing an S-phase entry assay, we found that 4-NP slightly, but significantly, reduced the number of cells incorporating BrdU (Figure 2A). The effect was not dose-dependent as we observed similar effects when 4-NP was used at 1, 10, or 25 μM. However, these experiments indicated that even low doses of 4-NP were able to decrease the proliferating ability of intestinal cells. To further investigate whether 4-NP was able to interfere with proliferative signals, cells were first treated with 0.1% serum-containing medium, to reduce the number of proliferating cells, then cells were induced to proliferate by adding EGF, in the absence or in the presence of 4-NP. As expected, EGF strongly stimulated cells to incorporate BrdU, but in the presence of 4-NP the ability of EGF to induce cells into S-phase was drastically reduced, also at low concentrations of 4-NP (Figure 2B).

4-NP reduced EGF-induced ERK-phosphorylation

Being ERK a downstream effector of EGF signalling, whose phosphorylation level rapidly increases after EGF stimulation, we analyzed the effect of 4-NP on EGF-induced ERK phosphorylation. In cells cultured with 0.1% serum-containing medium, EGF induced a marked increase of the level of p-ERK, as expected (Figure 3A and B). We also observed that the vehicle (DMSO 0.05%) caused a slight effect on basal ERK phosphorylation and also a slight but significant reduction of EGF-induced ERK phosphorylation, while 4-NP by itself did not cause any significant variation of ERK phosphorylation with respect to the untreated sample (Figure 3A and B). Interestingly, when we performed a brief pre-treatment with 25 μM 4-NP before adding EGF, we observed a remarkable reduction of ERK phosphorylation with respect to the level of phosphorylation due to EGF plus vehicle. Densitometric analyses showed that such a reduction was about three-fold (Figure 3A and B). These findings indicated that 4-NP interfered, at some level, with EGF-induced signalling, and confirmed the ability of 4-NP to reduce the effect of a proliferative stimulus.

Exposure to 4-NP induced apoptosis

It has been reported that 4-NP could trigger an apoptotic program in different cells and tissues (Jubendradass et al., 2012; Manente et al., 2011; Mao et al., 2008). We investigated whether human

intestinal epithelial cells could be sensible to induction of apoptosis after exposure to different concentrations of 4-NP for 24 h. By treating cells with etoposide, a well-known apoptosis-inducer, a number of apoptotic nuclei were evident after Hoechst staining. Similarly, when we treated cells with 1 and 10 μM 4-NP for 24 h, we observed the appearance of compact chromatin typical of apoptotic morphology in several microscopic fields (Figure 4A). We also performed a TUNEL assay to detect the occurrence of DNA fragmentation. We observed that both 1 μM and 10 μM 4-NP, as well as etoposide, induced a similar staining pattern (Figure 4B). Finally, to obtain a quantitative measure of the occurrence of apoptosis, we performed a caspase-3 assay to detect the biochemical activation of the apoptosis program. Indeed, the active form of caspase-3 is a crucial mediator of apoptosis (Porter and Jänicke,1999). As shown in figure 5, treatments with 4-NP for 24 h induced a dose-dependent effect on caspase-3 activity which reached a two-fold increase, with respect to basal activity, in cells treated with 50 μM of 4-NP. Etoposide was used as positive control in these experiments and caused an almost three-fold increase, with respect to basal activity.

4-NP induced ER-stress

Literature data indicated that 4-NP was able to mobilize calcium ions from ER, thus inducing an ER-stress response and lastly the activation of apoptosis program in rat neuronal and testicular cells (Gong et al., 2009; Kusunoki et al., 2007). We investigated whether treatments with 4-NP also induced ER-stress in Caco-2 cells. To this aim, we monitored the appearance of a well known ER-stress marker, i.e. the spliced form of XBP1 (Chakrabarti et al., 2011). When we treated cells with 4-NP for 60 min, we observed the appearance of the spliced form of XBP1, already at the concentration of 10 μM (Figure 6). The intensity of the band of the spliced form increased at higher concentration of 4-NP. In these experiments the ER-stress inducer THP served as positive control. XBP1 splicing was not a transient event, as we also found the spliced form of XBP1 after 5 h of treatment with 4-NP (data not shown).

We also evaluated whether 4-NP was able to induce an increase of GRP78 expression, being GRP78 an ER-resident chaperon whose expression is strongly increased during the unfolded protein response (UPR) triggered to resolve ER-stress (Lee, 2001). We performed a semi-quantitative analysis of the expression of GRP78 gene by real time RT-PCR. We found that treatments for 5 h with 10 μM 4-NP induced a two-fold increase of GRP78 mRNA expression, while at a higher 4-NP concentration (100 μM) we observed almost a 10-fold increase with respect to untreated or vehicle-treated cells (Figure 7). As expected, THP caused a 20-fold increase of GRP78 expression with respect to untreated cells. To confirm that 4-NP induced GRP78 expression, we analyzed GRP78 protein level by western blot on lysates obtained from cells treated with 4-NP for 24 and 72 h

(Figure 8A and B). We found a significant increase of GRP78 protein level after 24 h of treatments with 4-NP 50 and 100 μM. We observed a significant increase of the protein level also at 4-NP 10 and 25 μM after longer treatments (72 h), indicating that prolonged stimuli with 4-NP determined the persistence of the ER-stress response. As expected, THP induced a marked increase of GRP78 protein level already at 24 h of stimulation (Figure 8A).

Discussion

Several recent works have demonstrated that the exposure to the ubiquitous pollutant 4-NP could display a wide range of harmful effects on human health. Indeed, it may enhance the allergic response (Suen et al., 2012) and interfere with immune function (Bennasroune et al., 2012). It may contribute to the induction of metabolic syndrome (Wada et al., 2007) and may also affect male fertility (Lagos-Cabrè and Moreno, 2012) and pregnancy (Bechi et al., 2013). Moreover, since 4-NP displays a low estrogenic activity, it may potentially affect the development of hormone-dependent breast cancer (Recchia et al., 2004).

Even if the gastrointestinal tract represents the main route by which 4-NP enters into the body, up to date only few studies have been conducted on intestinal study models. It has been suggested that also low daily ingested doses of 4-NP may represent a risk for gut homeostasis because 4-NP tends to accumulate in gastrointestinal tissues. To this regard, available data on mammals are relative to studies on metabolism and dynamics of elimination of 4-NP in rat intestine. Daidoji et al. (2006) showed that, after oral administration of 4-NP, a high level of glucuronidated 4-NP was found in rat intestine. Moreover, they found that both 4-NP and its glucuronide were poorly excreted and persisted for long periods in the gastrointestinal tissue, leading to accumulation and subsequent release to other organs (Daidoji et al., 2006). Another important aspect to consider is the potential damaging effect that 4-NP may exert on probiotics normally hosted in the human gastrointestinal tract and important for its functionality. Regarding this, a recent study demonstrated that 4-NP dose-dependently inhibited growth of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, thus being able to interfere with normal intestinal microbiota and potentially contribute to an increased incidence of gastrointestinal disorders (Hsu et al., 2009).

Previous studies on differentiated Caco-2 cells, a human intestinal epithelial cell line considered a good *in vitro* model of human enterocytes (Meunier et al., 1995), indicated that 4-NP was able to affect cell barrier function. Indeed, treatments with low concentrations of 4-NP reversibly opened tight junctions and reduced transepithelial electrical resistance values of cell monolayers (Doo et al., 2005). Such effects became irreversible when treatment with 4-NP reached 72 h (Doo et al., 2005). A proteomic study showed that Caco-2 cells treated with 4-NP for 6 days over expressed proteins

(i.e. peroxiredoxin-1, glutathione S-transferase A2, galectin-3), which are possibly involved in the protective response towards toxic agents (Isoda et al., 2006). Finally, a recent study reported that low doses of 4-NP induced a pro-inflammatory response in the intestinal COLO320DM cell line (Kim et al., 2014).

Here we report findings about the toxic effect of 4-NP in undifferentiated Caco-2 cells, i.e. cells cultured for few days before treatments. At this stage, Caco-2 cells resemble intestinal crypt epithelial cells (Grasset et al., 1984), thus representing a suitable model to study cell growth and viability. We demonstrated that 4-NP was cytotoxic at relatively low concentrations. Indeed, we observed a reduction of cell number after 24 h of treatment already in the presence of 4-NP 10 μM and a decrement of mitochondrial functionality already in the presence of 4-NP 1 μM. Cytotoxic effect of 4-NP partly implied cell death through necrosis and partly was responsible of the induction of the apoptosis program, as revealed by the appearance of typical apoptotic condensed nuclei and DNA fragmentation in cells treated with low doses of 4-NP. The analyses of caspase-3 activation revealed that 50 μM 4-NP induced an about two-fold increase of caspase-3 basal activity, thus confirming the occurrence of the apoptotic program.

Some studies have established that 4-NP-induced apoptosis was mediated by an ER-stress response (Gong et al., 2009; Kusunoki et al., 2007). This effect seemed to be related to 4-NP ability to block the ER calcium pumps, which normally removes calcium from cytoplasm, thus depleting calcium from ER (Gong et al., 2009; Hughes et al., 2000). The consequent ER dysfunction can lead to protein misfolding and ER-stress, that in turn triggers a series of signalling and transcriptional events known as UPR. The UPR attempts to restore homeostasis in the ER but, if unsuccessful, can trigger apoptosis in the stressed cells (Chakrabarti and Chen, 2011). We found that Caco-2 cells treated with 4-NP for 1 and 5 h showed the presence of the spliced and active form of XBP1, indicating that an UPR program has been triggered. To confirm the occurrence of the UPR-induced by 4-NP, we monitored the expression level of GRP78. Both GRP78 gene expression and protein level were up-regulated after 24 h of treatment. The protein level was further increased after 72 h of treatment, indicating the persistence of the ER-stress response when cells were exposed to 4-NP for prolonged time.

Literature data indicated that 4-NP is not only cytotoxic but it can also affect cell growth by reducing cell proliferating rate (Kudo et al., 2004; Manente et al., 2011; Qi et al., 2013). We observed this phenomenon in Caco-2 cells, too. By performing an S-phase entry assay we found that 4-NP slightly but significantly reduced the number of Caco-2 cells progressing into the cell cycle. We also demonstrated that 4-NP was able to reduce the proliferative effect induced by EGF, as well as to prevent the EGF-induced activation of ERK, a specific intracellular EGF effector.

Similarly, in a model of neuronal cells, 4-NP was able to reduce the nerve growth factor-induced ERK-phosphorylation (Kusunoki et al., 2008). Interestingly, the ability of 4-NP to derange growth factor signalling could consequently attenuate cell survival and promote the intrinsic apoptotic pathway (Allan and Clarke, 2009).

Conclusions

In conclusion, we demonstrated that 4-NP negatively affect cell viability and surviving of human intestinal cells, inducing cell death and ER-stress, as well as a down-regulation of the proliferative pathway. Altogether, our data delineate a scenario in which chronic exposure to 4-NP, due to the consumption of contaminated food or water, may lead to a local damage to cells of intestinal mucosa, with still unknown and potentially harmful consequences for human health.

[1]Abbreviations

4-NP, 4-nonylphenol; ER, endoplasmic reticulum; THP, thapsigargin; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; EGF, epidermal growth factor; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling; XBP1, X-box binding protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRP78, 78 kDa glucose-regulated protein; ERK, extracellular signal-regulated kinase; UPR, unfolded protein response.

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References

Ademollo N, Ferrara F, Delise M, Fabietti F, Funari E. Nonylphenol and octylphenol in human breast milk. Environ Int 2008;34:984-7. doi: 10.1016/j.envint.2008.03.001.

Allan LA, Clarke PR. Apoptosis and autophagy: Regulation of caspase-9 by phosphorylation. FEBS J 2009;276(21):6063-73. doi: 10.1111/j.1742-4658.2009.07330.x.

Balakrishnan B, Thorstensen E, Ponnampalam A, Mitchell MD. Passage of 4-nonylphenol across the human placenta. Placenta 2011;32:788-92. doi: 10.1016/j.placenta.2011.07.014.

Bechi N, Sorda G, Spagnoletti A, Bhattacharjee J, Vieira Ferro EA, de Freitas Barbosa B, et al. Toxicity assessment on trophoblast cells for some environment polluting chemicals and 17β-estradiol.Toxicol In Vitro 2013;27(3):995-1000. doi: 10.1016/j.tiv.2013.01.013.

Bennasroune A, Rojas L, Foucaud L, Goulaouic S, Laval-Gilly P, Fickova M, et al. Effects of 4-nonylphenol and/or diisononylphthalate on THP-1 cells: impact of endocrine disruptors on human immune system parameters. Int J Immunopathol Pharmacol 2012;25(2):365-76.

Capaldo A, Gay F, Valiante S, De Falco M, Sciarrillo R, Maddaloni M, et al. Endocrine-disrupting effects of nonylphenol in the newt, *Triturus carnifex* (Amphibia, Urodela). Comp Biochem Physiol C Toxicol Pharmacol 2012;155(2):352-8. doi: 10.1016/j.cbpc.2011.10.004.

Caputo I, Barone MV, Lepretti M, Martucciello S, Nista I, Troncone R, et al. Celiac anti-tissue transglutaminase antibodies interfere with the uptake of alpha gliadin peptide 31-43 but not of peptide 57-68 by epithelial cells. Biochim Biophys Acta 2010;1802(9):717-27. doi: 10.1016/j.bbadis.2010.05.010.

Caputo I, Secondo A, Lepretti M, Paolella G, Auricchio S, Barone MV, et al. Gliadin peptides induce tissue transglutaminase activation and ER-stress through Ca2+ mobilization in Caco-2 cells. PLoS One 2012;7(9):e45209. doi: 10.1371/journal.pone.0045209.

Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011;108(12):2777-93. doi: 10.1002/bit.23282.

Daidoji T, Ozawa M, Sakamoto H, Sako T, Inoue H, Kurihara R, et al. Slow elimination of nonylphenol from rat intestine. Drug Metab Dispos 2006;34(1):184-90.

Doo MH, Li H, Jang HI, Song IS, Chung SJ, Shim CK. Effect of nonylphenol ethoxylates (NPEs) on barrier functions of epithelial cell membranes: opening of tight junctions and competitive inhibition of P-gp-mediated efflux. Int J Pharm 2005;302(1-2):145-53.

Ferrara F, Fabietti F, Delise M, Funari E. Alkylphenols and alkylphenol ethoxylates contamination of crustaceans and fishes from the Adriatic Sea (Italy). Chemosphere 2005;59(8):1145-50.

Gong Y, Wu J, Huang Y, Shen S, Han X. Nonylphenol induces apoptosis in rat testicular Sertoli cells via endoplasmic reticulum stress. Toxicol Lett 2009;186(2):84-95. doi:10.1016/j.toxlet.2009.01.010.

Grasset E, Pinto M, Dussaulx E, Zweibaum A, Desjeux JF. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. Am J Physiol 1984;247(3 Pt 1):C260-7.

Guenther K, Heinke V, Thiele B, Kleist E, Prast H, Raecker T. Endocrine disrupting nonylphenols are ubiquitous in food. Environ Sci Technol 2002;36(8):1676-80.

Hong L, Li MH. Acute toxicity of 4-nonylphenol to aquatic invertebrates in Taiwan. Bull Environ Contam Toxicol 2007;78(6):445-9.

Hsu WT, Lin TH, Chang EE, Hung CH, Huang AL, Wu T, et al. The effect of nonylphenol on the growth of Lactobacillus acidophilus and Bifidobacterium bifidum. J Microbiol Immunol Infect 2009;42(6):451-6.

Hughes PJ, McLellan H, Lowes DA, Kahn SZ, Bilmen JG, Tovey SC, et al. Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca(2+) pumps. Biochem Biophys Res Commun 2000;277(3):568-74.

Isoda H, Talorete TP, Han J, Nakamura K. Expressions of galectin-3, glutathione S-transferase A2 and peroxiredoxin-1 by nonylphenol-incubated Caco-2 cells and reduction in transepithelial electrical resistance by nonylphenol. Toxicol In Vitro 2006;20(1):63-70.

Jie X, Jianmei L, Zheng F, Lei G, Biao Z, Jie Y. Neurotoxic effects of nonylphenol: a review. Wien Klin Wochenschr 2013;125(3-4):61-70. doi: 10.1007/s00508-012-0221-2.

Jubendradass R, D'Cruz SC, Rani SJ, Mathur PP. Nonylphenol induces apoptosis via mitochondria- and Fas-L-mediated pathways in the liver of adult male rat. Regul Toxicol Pharmacol 2012;62(3):405-11. doi: 10.1016/j.yrtph.2012.01.004.

Kim A, Jung BH, Cadet P. A novel pathway by which the environmental toxin 4-Nonylphenol may promote an inflammatory response in inflammatory bowel disease. Med Sci Monit Basic Res 2014;20:47-54. doi: 10.12659/MSMBR.890644.

Kudo C, Wada K, Masuda T, Yonemura T, Shibuya A, Fujimoto Y, et al. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J Neurochem. 2004;88(6):1416-23.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 1998; 139:4252-63.

Kusunoki T, Shimoke K, Komatsubara S, Kishi S, Ikeuchi T. p-Nonylphenol induces endoplasmic reticulum stress-mediated apoptosis in neuronally differentiated PC12 cells. Neurosci Lett 2008;431(3):256-61.

Lagadic L, Coutellec MA, Caquet T. Endocrine disruption in aquatic pulmonate molluscs: few evidences, many challenges. Ecotoxicology 2007;16(1):45-59.

Lagos-Cabré R, Moreno RD. Contribution of environmental pollutants to male infertility: a working model of germ cell apoptosis induced by plasticizers. Biol Res 2012;45(1):5-14. doi: 10.4067/S0716-97602012000100001.

Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 2000;54(1):154-67.

Lee. The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 2001;26(8): 504-10.

Lorenc JF, Lambeth G, Scheffer W. Alkylphenols. In: Kroschwitz JI, Howe-Grant M, editors. Kirk-Othmer encyclopedia of chemical technology. New York: John Wiley and Sons; 1992. vol. 2, p. 113-143. doi: 10.1002/0471238961.0112112512151805.a01.pub2

Lu YY, Chen ML, Sung FC, Wang PSG, Mao IF. Daily intake of 4-nonylphenol in Taiwanese. Environ Int 2007;33:903-10. doi: 10.1016/j.envint.2007.04.008

Manente L, Sellitti A, Lucariello A, Laforgia V, De Falco M, De Luca A. Effects of 4-nonylphenol on proliferation of AGS gastric cells. Cell Prolif 2011;44(5):477-85. doi: 10.1111/j.1365-2184.2011.00774.x.

Mao Z, Zheng YL, Zhang YQ, Han BP, Chen LT, Li J, et al. Chronic application of nonylphenol-induced apoptosis via suppression of bcl-2 transcription and up-regulation of active caspase-3 in mouse brain. Neurosci Lett 2008;439(2):147-52. doi: 10.1016/j.neulet.2008.05.006.

Meunier V, Bourrié M, Berger Y, Fabre G. The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. Cell Biol Toxicol 1995;11(3-4):187-94.

Mosconi G, Carnevali O, Franzoni MF, Cottone E, Lutz I, Kloas W, et al. Environmental estrogens and reproductive biology in amphibians. Gen Comp Endocrinol 2002;126(2):125-9.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.

Popek W, Dietrich G, Glogowski J, Demska-Zakeś K, Drag-Kozak E, Sionkowski J, et al. Influence of heavy metals and 4-nonylphenol on reproductive function in fish. Reprod Biol 2006;6 Suppl 1:175-88.

Porter AG, Jänicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999;6(2):99-104.

Qi Y, Zhang Y, Liu Y, Zhang W. Nonylphenol decreases viability and arrests cell cycle via reactive oxygen species in Raji cells. Exp Toxicol Pathol 2013;65(1-2):69-72. doi: 10.1016/j.etp.2011.06.002.

Raecker T, Thiele B, Boehme RM, Guenther K. Endocrine disrupting nonyl- and octylphenol in infant food in Germany: considerable daily intake of nonylphenol for babies. Chemosphere 2011 82(11):1533-40. doi: 10.1016/j.chemosphere.2010.11.065.

Recchia AG, Vivacqua A, Gabriele S, Carpino A, Fasanella G, Rago V, et al. Xenoestrogens and the induction of proliferative effects in breast cancer cells via direct activation of oestrogen receptor alpha. Food Addit Contam 2004;21(2):134-44.

Shanle EK, Xu W. Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. Chem Res Toxicol 2011;24(1):6-19. doi: 10.1021/tx100231n.

Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ Int 2008;34(7):1033-49. doi: 10.1016/j.envint.2008.01.004.

Suen JL, Hung CH, Yu HS, Huang SK. Alkylphenols--potential modulators of the allergic response. Kaohsiung J Med Sci. 2012 Jul;28(7 Suppl):S43-8. doi: 10.1016/j.kjms.2012.05.009.

Terasaka S, Inoue A, Tanji M, Kiyama R. Expression profiling of estrogen-responsive genes in breast cancer cells treated with alkylphenols, chlorinated phenols, parabens, or bis- and benzoylphenols for evaluation of estrogenic activity. Toxicol Lett 2006;163:130.

Wada K, Sakamoto H, Nishikawa K, Sakuma S, Nakajima A, Fujimoto Y, et al. Life style-related diseases of the digestive system: endocrine disruptors stimulate lipid accumulation in target cells related to metabolic syndrome. J Pharmacol Sci 2007;105(2):133-7.

Legend to figures

Figure 1 - Effect of 4-NP on cells viability (A). Percent of decrease of living cells as a consequence of 4-NP treatments (dose range 1-100 μM) for 24 h. **(B)** Percent of reduction of cell viability measured by the MTT assay performed on cells treated with 4-NP (dose range 1-100 μM) for 24 h. H_2O_2 represented the positive control. Data were reported as mean ± SD of three independent experiments. *p<0.05 *vs.* untreated and DMSO-treated cells.

Figure 2 - Effect of the treatment with 4-NP on BrdU incorporation by Caco-2 cells (A) Reduction of BrdU incorporation by cells cultured for 18 h in the presence of 1, 10 and 25 μM 4-NP. **(B)** Dose-dependent inhibition of EGF-induced BrdU incorporation by cells cultured for 18 h in the presence of 1, 10 and 25 μM 4-NP. Data were expressed as mean ± SD of three independent experiments. *p<0.05 vs. untreated and DMSO-treated cells. Inserts are representative microscopic fields, x40, of nuclei stained with Hoechst (up) and with TRITC-conjugated anti-BrdU antibody (down), showing the effect of treatment with EGF plus 25 μM 4-NP.

Figure 3 - Effect of 4-NP on ERK phosphorylation. (A) Western blot analyses on total proteins (75 μg), stained by ERK or p-ERK antibodies, showed an increase of ERK phosphorylation after 5 min of EGF treatment (with or without vehicle) and a less pronounced increase after 5 min of EGF treatment in the presence of 4-NP 25 μM. The blot was representative of three independent experiments. The appearance of two bands is relative to the presence of two closely related kinases, ERK 1 (upper) and ERK 2 (lower). **(B)** Densitometric analysis (mean of three independent western blot experiments) of p-ERK level. The analysis was performed on ERK 2 bands. The amount of p-ERK was normalized to the amount of total ERK. *p<0.05 relative to EGF/DMSO *vs.* EGF. **p<0.05 relative to EGF/25 μM 4-NP vs. EGF/DMSO.

Figure 4 - Visualization of cell apoptosis induced by 4-NP. (A) Microscopic visualization, x40 with oil, of representative Hoechst stained nuclei of Caco-2 cells treated with 1 and 10 μM 4-NP. White arrows indicated nuclei in which apoptotic morphology was evident. **(B)** Representative microscopic images, x40, concerning the TUNEL assay on Caco-2 cells treated with 1 and 10 μM 4-NP for 24 h. Black arrows indicated nuclei in which DNA fragmentation was present. Both in (A) and (B), etoposide was used as positive control.

Figure 5 - Caspase-3 activity in cells treated with 4-NP. Dose-dependent activation of caspase-3 activity in the presence of 4-NP, quantified using a colorimetric assay after 24 h of treatment. Etoposide was used as positive control. Results were expressed as mean ± SD of three independent experiments. *p<0.05 *vs.* untreated and DMSO-treated cells.

Figure 6 - Induction of XBP1 splicing in cells treated with 4-NP. Treatments with 4-NP 10, 50 and 100 μM for 1 h induced the appearance of the spliced form of XBP1 on a 2% agarose gel stained with ethidium bromide. cDNA amplification of the house-keeping gene GAPDH was also shown.

Figure 7 - Analysis of 4-NP-induced GRP78 gene expression in Caco-2 cells. Semi-quantitative analysis of GRP78 mRNA by real-time RT-PCR after 5 h of treatment with 4-NP 10, 50, and 100 μM or with THP. The amount of mRNA of GRP78 was normalized to that of GAPDH. Values are the mean ± SD of three independent experiments. *$p<0.05$ *vs.* untreated and DMSO-treated cells.

Figure 8 - Analysis of 4-NP-induced GRP78 protein expression in Caco-2 cells. (A) Western blot analyses on total proteins (75 μg) of GRP78 protein level after 24 and 72 h of treatment with increasing amounts of 4-NP. Cells were exposed to THP for 24 h only. The blots shown were representative of three independent experiments. **(B)** Densitometric analysis of protein levels after 24 and 72 h of treatments. The amount of GRP78 is normalized to that of GAPDH. Values are the mean ± SD of three independent experiments. *$p<0.05$ *vs.* DMSO-treated cells.

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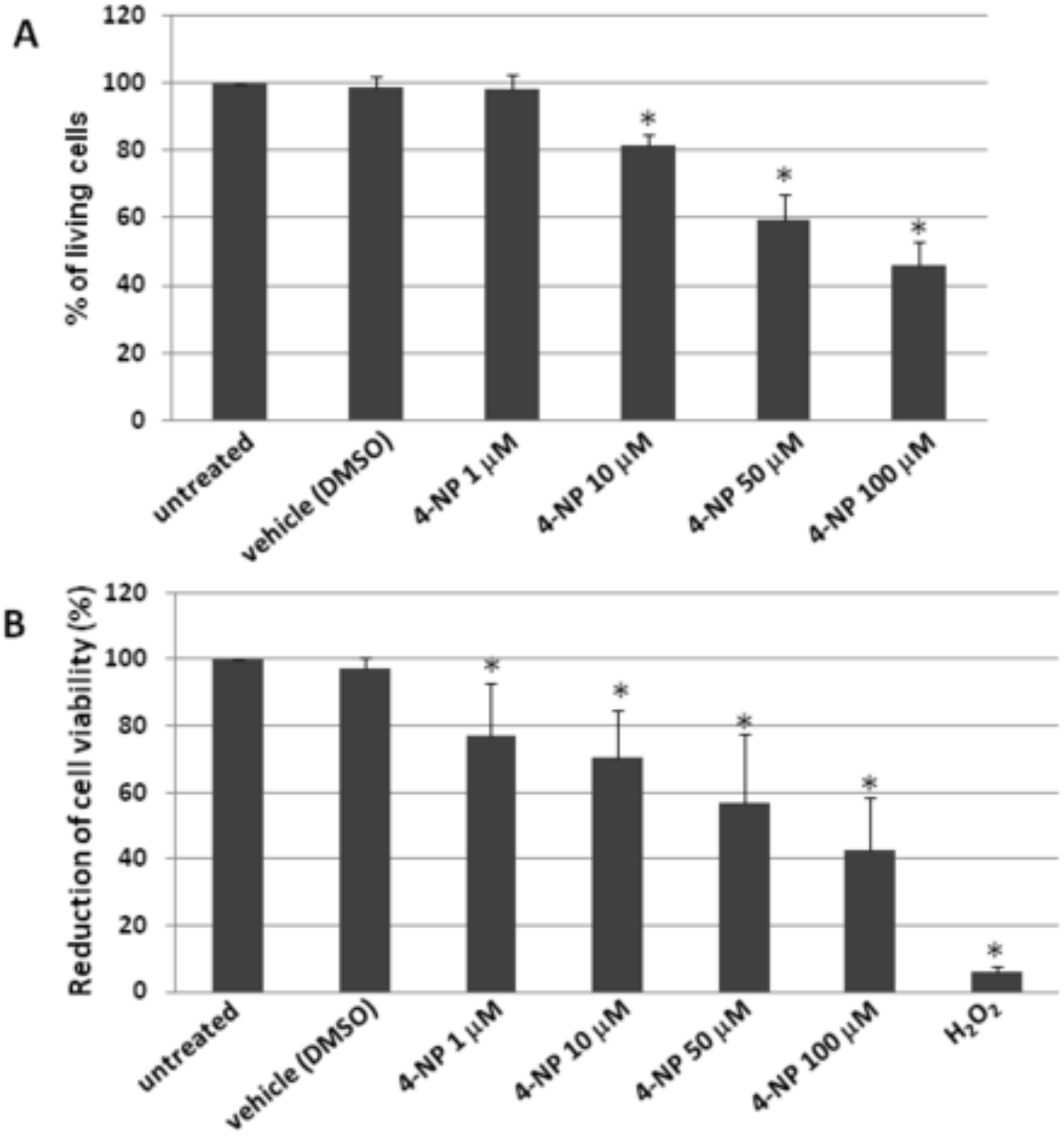

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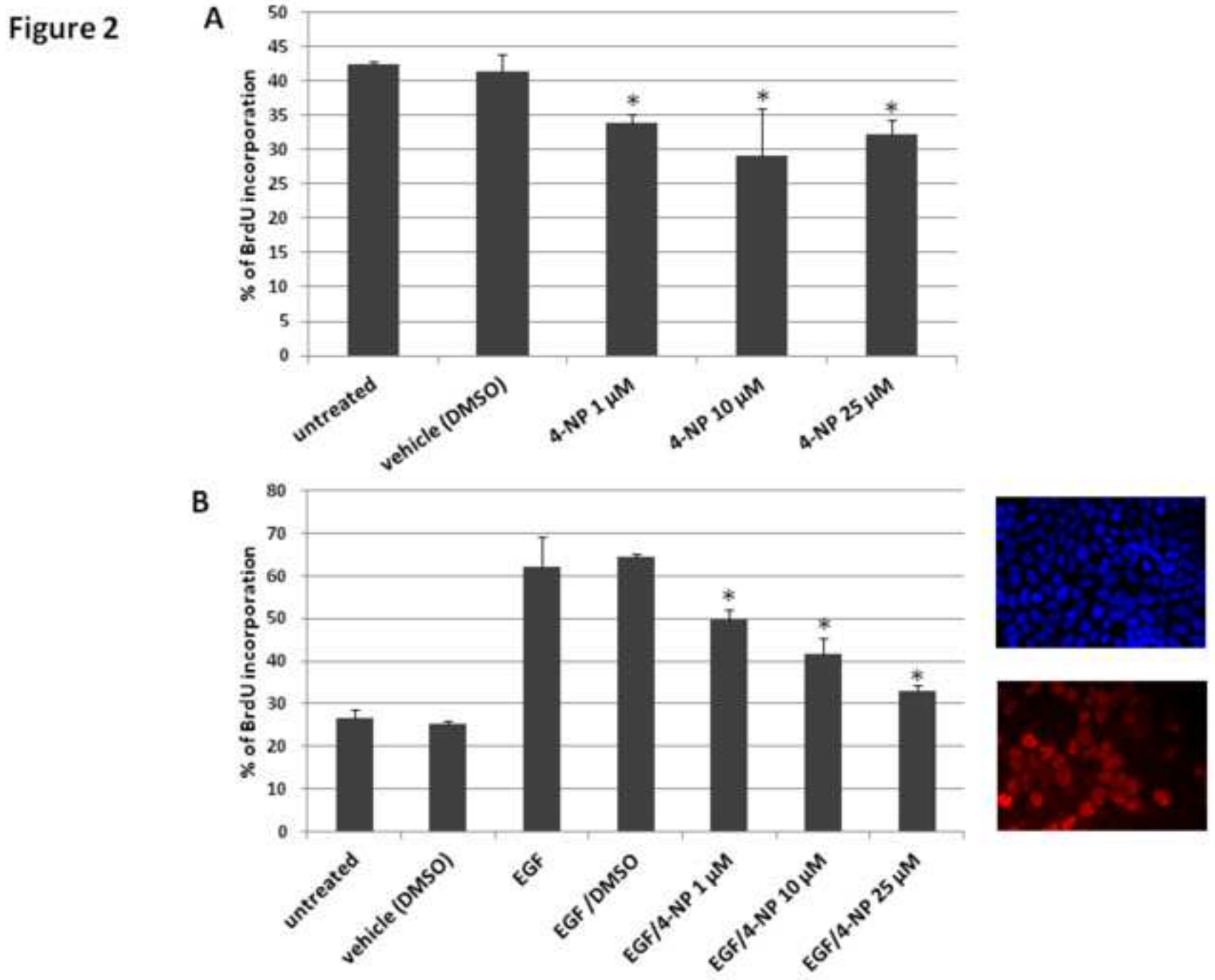

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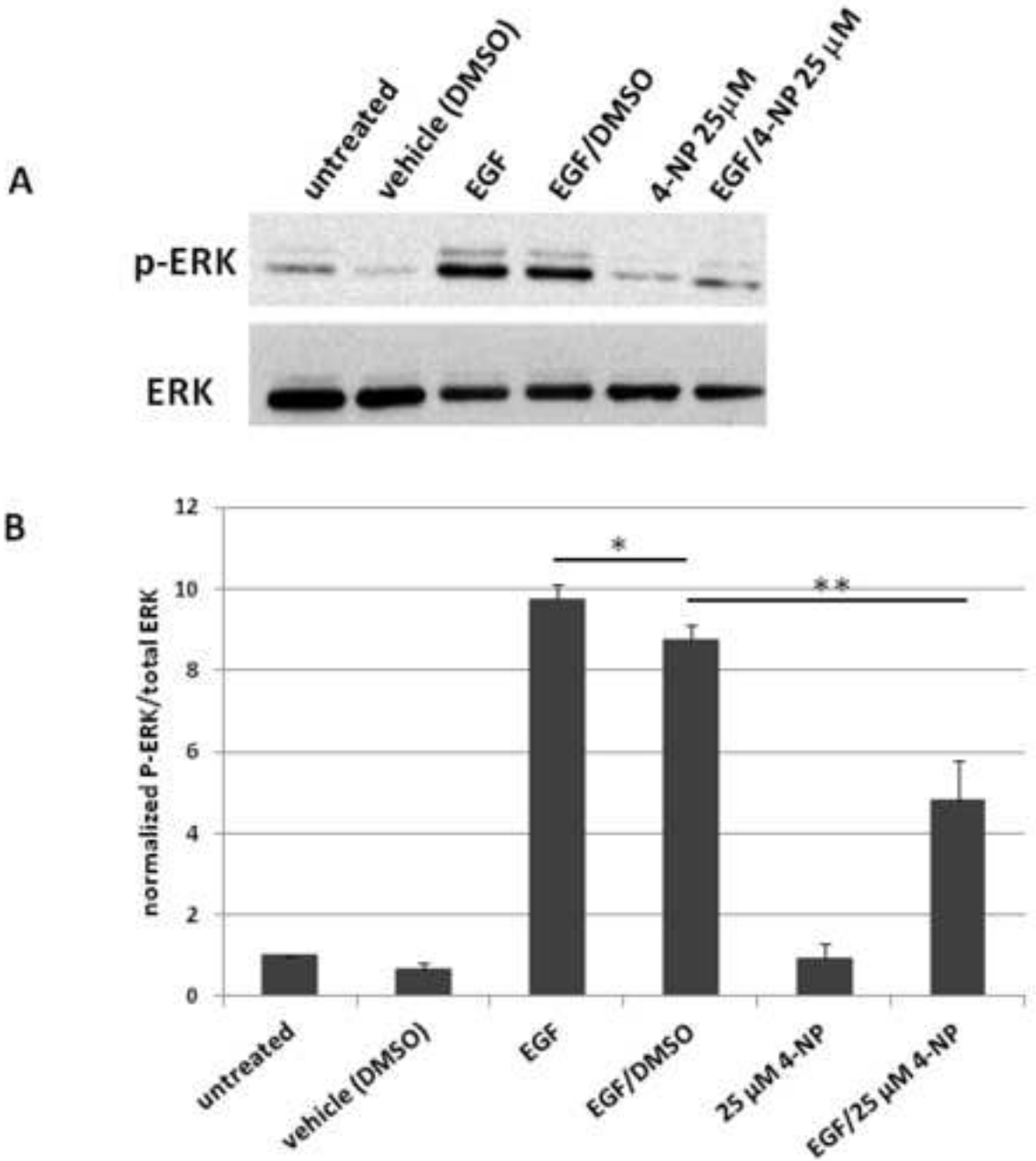

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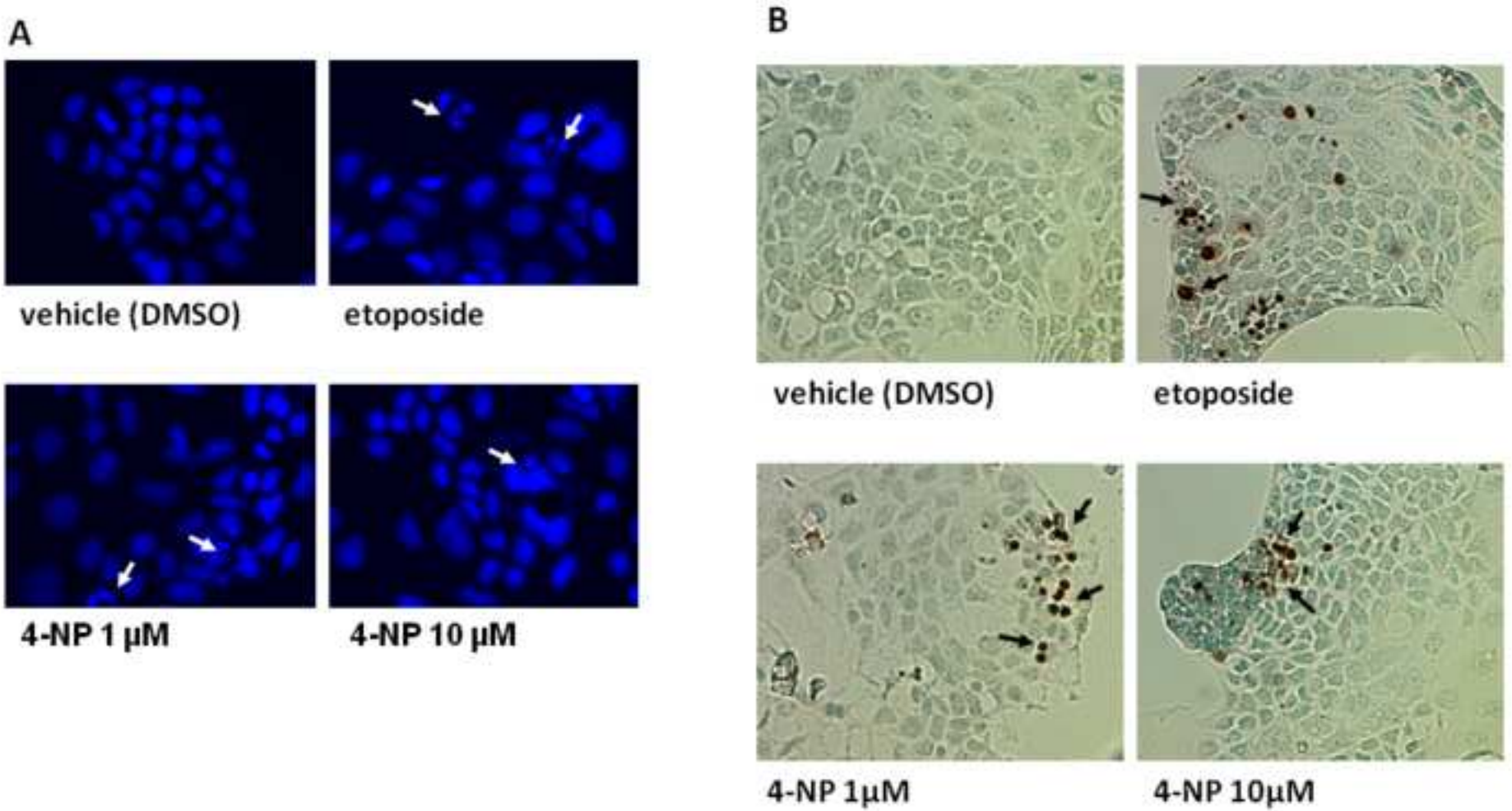

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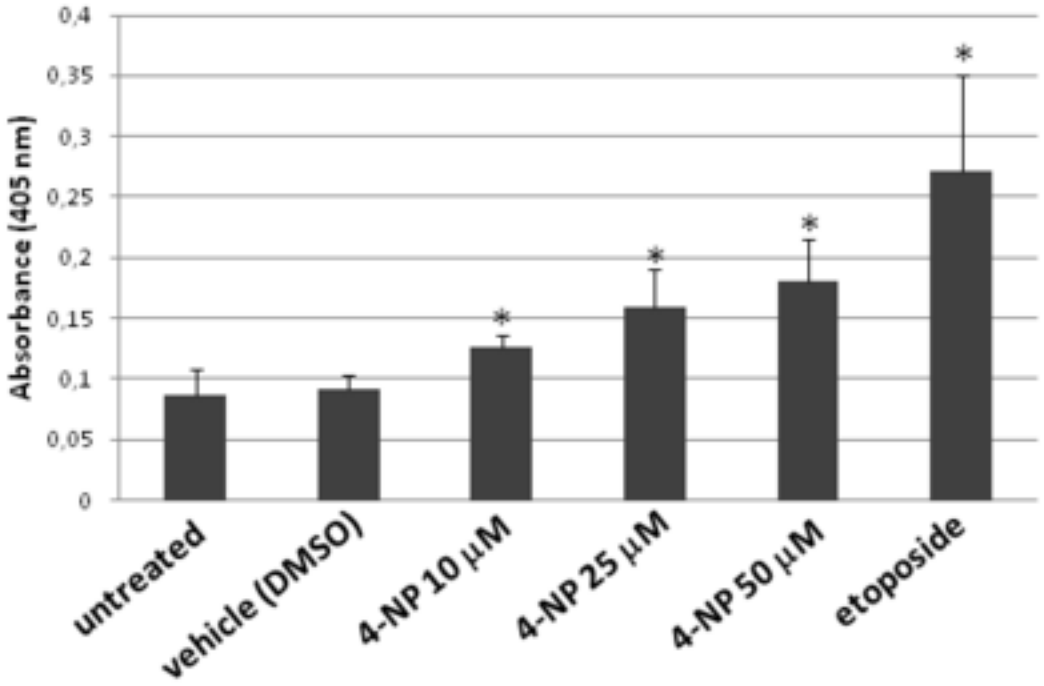

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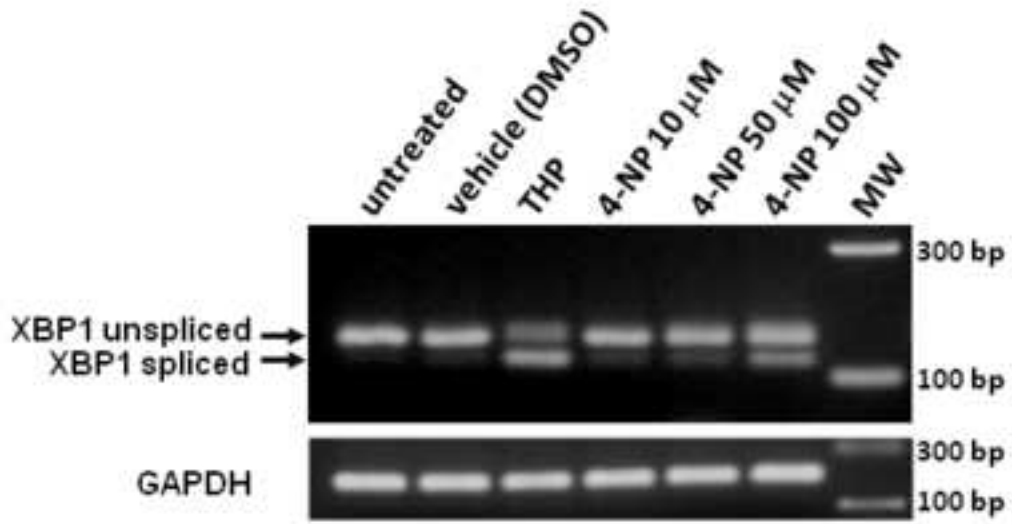

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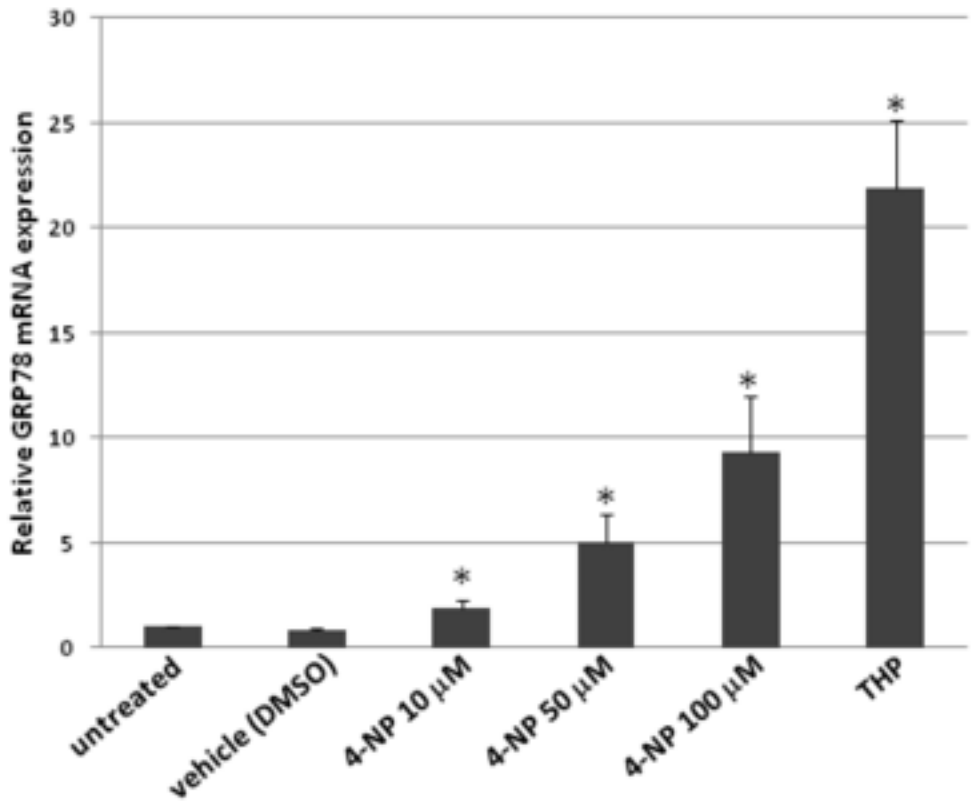

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