Diisononyl Phthalate differentially affects sirtuin expression in the HepG2 cell line

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Abstract

Human exposure to phthalates has received especial attention due to their possible adverse human health effects. Diisononyl phthalate (DINP) is a plasticizer still widely used in many products, despite being considered an endocrine disruptor. In this study, we evaluated DINP's cytotoxicity, its effect on the levels of reactive oxygen species (ROS), and its effect on sirtuin expression in HepG2 cells. Results showed that 1 µg/mL DINP significantly downregulated Sirt1, Sirt2, Sirt3, and Sirt5 gene expression (p<0.05), while other sirtuins remained unaffected. Furthermore, protein levels of Sirt1 and Sirt3 were significantly downregulated by 1 µg/mL DINP. On the other hand, 100 µg/mL DINP doubled the levels of lysine acetylation proteins (increased two-fold) as well as reactive oxygen species (ROS) compared with the controls. In conclusion, our study suggests, for the first time, that DINP regulates the potential epigenetic disruptor sirtuin family, and leads to induction of ROS via sirtuins.

Keywords: diisononyl phthalate; sirtuins; HepG2 cells; plasticizers

1. Introduction

Endocrine disrupting chemicals (EDCs) are defined as exogenous substances that have the ability to cause adverse health effects in an intact organism by altering functions of the endocrine system [1]. It was originally thought that EDCs act primarily through nuclear hormone receptors; however, it is now widely accepted that EDCs act through a variety of signaling mechanisms, which include nuclear steroid receptors, nonsteroid receptors, orphan receptors, epigenetic modifications, and enzymatic pathways ultimately responsible for maintaining endocrine homeostasis [2].

Phthalates are a group of endocrine disruptors used as plasticizers in materials such as polyvinyl chloride, along with being involved in the manufacturing processes of many other products. Diisononyl phthalate (DINP) is one of the primary phthalates most used in the industry. It is a mixture of compounds consisting of isononyl esters of phthalic acid (Fig. 1). DINP is widely used in flooring, wire and cable, dip coating, coated fabrics, tubing, shoes, sealants, and artificial leather; humans may be exposed to DINP by oral, dermal, and inhalation routes [3]. The environmental ubiquity of DINP is known and its presence has been even reported in river water, drinking water, outdoor air, and indoor air [4]. DINP and its metabolites are widely studied and have received considerable attention recently because of specific concerns about dietary or medical exposure in pediatric patients [5].

It has been proposed that population exposure to DINP would not exceed the levels of di-(2-ethylhexyl) phthalate (DEHP) [3], which are estimated at 3-30 μg/kg body weight/day [6]. Phthalates, including DINP, are not covalently bound to plastics and can migrate into saliva, where they are swallowed [3, 7]. Thus, children may be exposed to higher levels of DINP than adults are, because infants and small children mouth toys and other articles containing DINP [3, 8]. The chronic health effects of DINP, including organ toxicity, carcinogenicity, and reproductive toxicity, have been reviewed in dietary studies [3, 8].

Early life exposure to phthalates has been associated with a variety of adverse effects, particularly those involving endocrine processes [3, 9]. It has been noted that levels of phthalate metabolites in urine and serum are associated with central obesity and insulin resistance in adults [10, 11], suggesting that adult exposure to phthalates may link obesity with related metabolic disorders. This is in addition to a possible contributing role in the development of obesity, as shown by recent data reporting an association between urinary levels of phthalates and higher odds for obesity (body mass index) in children and adolescents [9].

Sirtuins (Sirts) are a group of mitochondrial NAD^+ dependent histone deacetylases which have emerged as key epigenetic regulators that act as cellular sensors by detecting energy availability and modulating metabolic processes [12]. Sirtuins are involved in several cellular functions including chromosomal stability, DNA repair, the cell cycle, apoptosis, metabolism, and aging by deacetylating a variety of transcription factors, histones, and non-histone proteins. Several studies identified Sirt3 as a potentially important factor in the pathogenesis of diabesity. For instance, Zhang et al. showed that butyl benzyl phthalate (BBP) decreases Sirt1

and Sirt3 gene expression and protein levels in HepG2 cells [13]. Additionally, our group also showed that several phathalates and persistent organic pollutants differentially modules sirtuin family in macrophage cells [14]. The aim of this study was to investigate sirtuin regulation, levels of lysine acetylation proteins, and the generation of reactive oxygen species under the exposure of diisononyl phthalate (DINP) by HepG2 cells.

2. Material and methods

2.1. Cell culture and exposure conditions

The human hepatocellular carcinoma cell line (HepG2) were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin/streptomycin (Sigma) at 37°C in 5% CO_2 in an incubator. For treatments with DINP (Sigma), the cells were cultured in 24 well plates ($2x10^5$ cells per well in 1 mL of medium) for 1 day before using them for exposition. HepG2 cells were cultured with various concentrations of DINP (0.1, 1, 10 and 100 µg/mL). After 48 h of treatment, mRNA was extracted and quantified for gene expression. To analyze protein expression of Sirt1, Sirt2, Sirt3, Sirt5 and acetylated proteins, HepG2 cells were cultured with the same DINP concentrations (0.1, 1, 10 and 100 µg/mL), and after 72 h of treatment, nuclear and mitochondrial proteins were extracted and analyzed by Western Blot. Briefly, the cells were harvested in ice-cold buffer B (containing 20mM HEPES, 1.5 mM $MgCl_2$, 25% of Glycerol, 420 mM NaCl, 0.5mM DTT, 0.2 mM EDTA) and supplemented with protease inhibitor cocktail and 0.5 mM PMSF (Sigma).

2.2. Cell Viability Assay

Cell viability was determined by the Tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using Thiazolyl Blue Tetrazolium Bromide powder (Sigma) as described previously [13]. Briefly, HepG2 cells were plated and incubated with different concentrations of DINP at 48 h. The cells were incubated with MTT solution (10 μL per well, 5 mg/mL in PBS) for 4 h at 37°C. The supernatant was then removed, and formazan crystals were dissolved in 100 μL of DMSO with orbital shaking. Optical densities of the resultant solutions were determined colorimetrically at 490 nm using a microplate reader (BioRad). The percentage of viable cells was determined by comparing the optical densities of cells incubated with the varying concentrations of DINP and DMSO control.

2.3. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted using TRI Reagent (Sigma). The cDNA was made from 1 μg of total RNA by using High- Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The forward and reverse primers used in the present study are shown in Table 1. Real-time PCR was performed using SYBR select master mix (Applied Biosystem). 18S cDNA level was used as a reference gene and the expression levels were normalized to 18S and gene expression was calculated using $2^{-\Delta\Delta CT}$ method and expressed as fold change [15]. All assays were carried out in triplicate.

2.4. Western blotting

Once the nuclear and mitochondrial proteins were extracted, the concentration was estimated with BSA reagents from Thermo Scientific. Equal amounts of protein were separated using SDS-PAGE and transferred to nitrocellulose membranes (Thermo Scientific). Membranes were blocked with 5% milk in TBS containing 0.1% Tween 20 (Sigma) for 1 h and then incubated with rabbit monoclonal antibodies anti Sirt1, Sirt2, Sirt3, Sirt5, Lysine-Acetylation and β-Actin (Cell Signaling Technology). AP conjugated (Sigma) secondary antibodies were used for detection and quantitation of immuno-blots. Membranes were developed using enzymatic substrate. Band densities were analyzed by ImageStudio software (LI-COR). All assays were made in triplicate.

2.5. Measurement of Reactive Oxygen Species

Reactive oxygen species levels (ROS) were measured using 2´, 7´-dichlorofluorescein diacetate (DCFDA, Sigma). Briefly, DINP-treated cells were seeded in a 96-well plate for 48 h. Cells were then incubated with 25 mM DCFDA for 45 min at 37ºC, and the fluorescence was measured using a plate reader (Fluoroskan Ascent FL, Thermo Scientific).

2.6. Statistical analysis

All data are presented as the mean ± standard error (S.E.). Statistical significance was determined by One-way ANOVA ($p < 0.05$). Pairwise comparisons were made using Tukey's test ($p < 0.05$).

3. Results

3.1. DINP affects cell viability in the HepG2 cell line

To determine the effect of DINP on cell viability, HepG2 cells were treated with different doses of DINP (0.1, 1, 10 and 100 µg/mL) for 48 h. The results showed that DINP has a dose-dependent effect on cell viability in HepG2 cell lines (Fig. 2). Low concentrations of DINP (0.1 and 1 µg/mL) showed insignificant cell death. Treatments with 10 and 100 µg/mL were significantly affected by DINP as compared to the control ($p<0.05$) and however 85% cells were viable for both concentrations.

3.2. DINP differentially regulates Sirtuin expression

The effect of DINP treatment on the gene expression of sirtuins was determined by treating HepG2 cells with different doses of DINP (0.1, 1, 10 and 100 µg/mL) for 48 h. Sirt1, Sirt2, and Sirt5 gene expression was significantly decreased at a low concentration (1 µg/mL) of DINP when compared to control ($p<0.05$). The gene expression levels of Sirt1, Sirt2 and Sirt5 at 1 and 10 µg/mL DINP decreased to 50%, while gene expression decreased by 75% with 100 µg/mL DINP. For Sirt3, the gene expression levels did not reach below 50% for all concentrations tested. Interestingly, gene expression of Sirt3 showed a significant decrease at 0.1 µg/mL

DINP ($p<0.001$) (Fig. 3), displaying a dose-dependent effect. Others sirtuins (Sirt 4, 6,7) evaluated remain unaffected.

3.3. DINP decreases Sirt1 and Sirt3 protein levels and increases acetylated protein in HepG2 cells

To determine the effect of DINP on Sirt1, Sirt2, Sirt3, Sirt5 and acetylated protein levels, HepG2 cells were treated with different doses of DINP (0.1, 1, 10 and 100 µg/mL) for 72 h. Treatments of 1 to 100 µg/mL DINP significantly decreased Sirt1 and Sirt3 protein levels (Fig. 4A). The protein expression levels of Sirt1 at 1 µg/mL DINP decreased 25% and at 100 µg/mL DINP decreased 50%. Similarly, protein levels of Sirt3 treated with 1 to 100 µg/mL DINP decreased to 50% compared to the control. On the other hand, 100 µg/mL DINP doubled the protein acetylation levels compared to the control (Fig. 4B). These results are consistent with the gene expression results. Interestingly, Sirt2 and Sirt5 did not show significant changes.

3.4. DINP treatment increases reactive oxygen species (ROS) levels

To investigate if DINP induced ROS production, HepG2 cells were treated with doses of DINP (0.1 to 100 µg/mL) for 48 h. The results showed that DINP increased the ROS levels in a dose-dependent manner and ROS levels were significantly increased ($p<0.05$) in the 100 µg/mL DINP treatments (Fig. 5).

4. Discussion

Several studies have shown that phthalates have toxic effects at levels similar to those to which average populations are currently exposed [16-18]. DINP has been identified as a food contaminant and it has been banned and restricted by regulatory agencies in the EU [19, 20]. Children may be particularly susceptible to the effects of DINP because they have higher relative exposures compared with adults (due to greater dietary intake per kilogram), their metabolic (*i.e.,* detoxification) systems are still developing, and key organ systems are undergoing substantial changes and maturations that are vulnerable to disruptions. In 2009-2010 NHANES data, it was reported that urinary metabolites of DINP were detected in 98% of the population. Additionally, cross-sectional data from NHANES from 2009 to 2012 show positive associations of DINP metabolite concentrations with insulin resistance and systolic blood pressure *z* scores in children and adolescents [21]. Therefore, in the present study, we investigated the possible effects of exposure to DINP phthalate on sirtuin gene and protein expression levels. Since phthalate exposure in humans is widespread, it is important to know if phthalates can interact with epigenetic regulators such as sirtuins, which can promote long-term changes in metabolic homeostasis, potentially leading to deleterious physiological consequences.

Our results showed, for the first time, that DINP decreased Sirt1, Sirt2 and Sirt3 and gene expression (Fig. 3) as well as Sirt1 and Sirt3 protein levels were downregulated in a dose-dependent manner (Fig. 4). The study by Zhang, Ali [13] discovered that when HepG2 cells were treated with benzyl butyl phthalate (BBP), Sirt1 and Sirt3 gene and protein expression were decreased, which agrees with our results. Reduced levels of Sirt1 and Sirt3 expression have been shown to

cause impaired metabolic function or age-related complications [22, 23]. Therefore, DINP induced down-regulation of Sirt1 and Sirt3 may have a plausible correlation to recent metabolic-related health concerns.

Oxidative stress is due to the continuous production of ROS, which imbalances the production of free radicals and the antioxidant system, and can usually induce oxidative damage such as DNA oxidation, protein oxidation, and lipid peroxidation [24, 25]. Thus, oxidative stress can be considered an overwhelming generation of reactive species or a general disruption of redox cellular homeostasis. It has been reported to play an important role in the pathogenesis of diabetes, asthma and other diseases [26-28]. ROS may function as regulators of cell signaling, which may impact the development of a metabolic disorder. The production of ROS is negatively associated with cell viability, energy metabolism and metabolic diseases, and it is already known that during conditions of metabolic stress such as obesity and metabolic syndrome, an oxidative stress environment is created [25]. Oxidative damage by release of ROS has been attributed to some phthalates, including DEHP. Recently, several studies have reported an association between exposure to phthalates including DINP and oxidative stress. For example, Liu et al. Liu, Jiang [29] observed that ROS levels were increased significantly by Mono-(2-ethylhexyl) phthalate (MEHP). Aly et al. [30] showed that Dibutyl phthalate (DBP) induced testicular toxicity by oxidative stress. MEHP induces apoptosis through ROS-mediated mitochondrial-dependent pathway in HUVEC cells [31]. Franken et al. [32] showed a highly significant association of phthalate exposure with oxidative stress via DEHP and its main metabolite MEHP. Kang et al. [33] showed that DINP contributes to the development of allergic asthma by promoting the elevation of

oxidative stress and activating the NF-Kβ signaling pathway. Similarly, our results showed that ROS production increased when cells are exposed to DINP (Fig. 5).

The toxic effects associated with altered levels of subcellular ROS are largely prevented by various antioxidants, many of which are regulated by sirtuins and appear to be an integral part of an important cellular defense mechanism against oxidative stress and ROS formation. Several studies support the idea that sirtuins play very important roles in maintaining proper cellular redox balance and seem to be protecting the body from the adverse effects of oxidative stress and associated diseases. Therefore, sirtuins have emerged as key players in regulating the antioxidative capacity of cells. For instance, several reports support the idea that Sirt1 can mediate an oxidative stress response, directly deacetylating several transcription factors that regulate antioxidant genes [34-37]. For example, sirtuins can regulate oxidative stress mainly through forkhead transcription factor (FOXO), which controls a variety of cellular processes including ROS production, DNA repair and apoptosis [35]. Results reported by Brunet et al [34], demonstrated that Sirt1 deacetylates FOXO1 and FOXO3a and increases cellular resistance to oxidative stress in HEK 293 cells, where FOXO deacetylation confers cell resistance to oxidative stress. During oxidative stress, Sirt1-FOXO3a interaction increased the transcription of stress resistant genes and decreased the expression of FOXO3a- dependent proapoptotic genes [34]. In our case, DINP indeed acetylated several proteins in HepG2 cells (Fig. 4c,d)

Sirt2 has a critical role in the modulation of the oxidative stress response. Sirt2 is a central regulator of the defense mechanism against ROS, and has been shown to

deacetylate and activate Forkhead box O3 (FOXO3a), a transcriptional activator of superoxide dismutase 2 (SOD2) which in turn reduces the ROS level [38].

On the other hand, Sirt3 is known to mediate the flow of mitochondrial oxidative pathways and plays an important role in the detoxification of ROS, and therefore regulates the production of ROS [39]. Sirt3 has been shown to mediate the deacetylation of enzymes that are responsible for the reduction of ROS, leading to protection against oxidative stress. For instance, Sirt3 has been shown to deacetylate and activate isocitrate dehydrogenase 2 (IDH2), SOD2 (by direct deacetylation and activation of the enzymatic function) [40-42] and catalase, all key enzymes in reducing the cellular levels of ROS [25, 43, 44]. Sirt3 is shown to activate antioxidant machinery in the mouse heart by inducing the expression of SOD2 and catalase through deacetylation of the transcription factor FOXO3a [43]. In addition, decreased Sirt3 levels have been found in human epidermal keratinocytes after ozone exposure, which was correlated with increased DNA damage, higher levels of cellular H_2O_2, and reduced SOD2 protein levels [45] and loss of Sirt3 has been shown to increase the production of ROS [46].

It has been reported that Sirt5 also desuccinylates and activates the ROS detoxifying enzyme superoxide dismutase 1 (SOD1) and, at least in the brain, regulates the SOD2 expression [47, 48]. These findings strengthen evidence that phthalates can activate oxidative stress via sirtuins (Fig. 6).

5. Conclusion

Our study suggests that DINP can alter the potential epigenetic disruptor sirtuin family, and thus lead to the induction of ROS via sirtuins. We observed a decrease

in gene expression and protein levels of Sirt1 and Sirt3 proteins after exposure to DINP at concentrations that do not affect HepG2 cell viability. This can be correlated with an increase in acetylated proteins, which as a result leads to an increase in ROS levels. However, considering the widespread exposure to DINP in the population, *in vivo* studies will be required to understand the effect of DINP on the regulation of sirtuins. Identifying the mechanism whereby phthalate exposure is associated with a metabolic syndrome remains an important area of research.

6. Acknowledgments

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Table 1. Gene primers used in this study

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
h18S (F4)	CTCTAGATAACCTCGGGCCG	GTCGGGAGTGGGTAATTTGC
hSirt1 (F2)	TAGCCTTGTCAGATAAGGAAGGA	ACAGCTTCACAGTCAACTTTGT
hSirt2 (F1)	ATCCACCGGCCTCTATGACAA	CGCATGAAGTAGTGACAGATGG
hSirt3 (F1)	GACATTCGGGCTGACGTGAT	ACCACATGCAGCAAGAACCTC
hSirt4 (F2)	GAATCGGGGATACCAGACTACA	GCCAGCCTACGAAGTTTCTCG
hSirt5 (F1)	GCCATAGCCGAGTGTGAGAC	CAACTCCACAAGAGGTACATCG
hSirt6 (F1)	CCCGGATCAACGGCTCTATC	GCCTTCACCCTTTTGGGGG
hSirt7 (F1)	CGTCCGGAACGCCAAATAC	GACGCTGCCGTGCTGATT

Figure captions

Fig. 1 Chemical structure of DINP

Diisononyl phthalate (DINP)

Fig. 2 DINP compound induced cytotoxicity in HepG2 cells.

HepG2 cells were incubated with different concentrations of DINP for 48 h. Cell viability was then determined by MTT assay. Two different (A) DMSO and (B) EtOH diluents were tested, demonstrating that neither causes an adjuvant or cytotoxic effect to the cell. Data represent the means ± S.E.M (n=6) * p <0.05, ** <0.01, versus untreated control.

Fig. 3 DINP decreased the expression of Sirt1, Sirt2 and Sirt3 in HepG2 cells.

HepG2 cells were exposed for 48 h at different concentrations (0.1, 1, 10 and 100 µg/mL) of DINP. (A-G) The mRNA levels of Sirts 1 to 7 were determined by qPCR. The 18S gene served as an endogenous control. All data are mean ± S.E.M. * p <0.05, ** <0.01 *** <0.001 compared to an untreated control; n = 3.

Fig. 4 Protein levels of Sirt1 and Sirt3 were affected at 1 µg/mL of DINP, and acetylation protein levels were increased.

HepG2 cells were exposed for 72 h at different concentrations (0.1, 1, 10 and 100 µg/mL) of DINP. (A-B) Protein levels of Sirt1, Sirt3 and (C-D) Lysine acetylation were determined by Western Blot analysis. Quantification of expression was

described as ratio of protein level to β-Actin level. One representative blot is shown. All data are mean ± S.E.M. * p <0.05, ** <0.01 compared to an untreated control; n = 3.

Fig. 5 Highest concentrations of DINP increased ROS levels in HepG2 cells

Reactive oxygen species (ROS) levels were determined by measuring oxidized dichlorofluorescein (DCF) levels using 2, 7- dichlorofluorescein diacetate (DCFDA). Data represent the mean ± standard error versus untreated control. * *p* <0.05, n= 4.

Fig. 6 Proposed model for the effect of DINP in sirtuin regulation

DINP treatment induces downregulation of sirtuins, which leads to increased levels of ROS production by acetylation of several proteins involved in the oxidative stress pathway and modulation of metabolic genes.

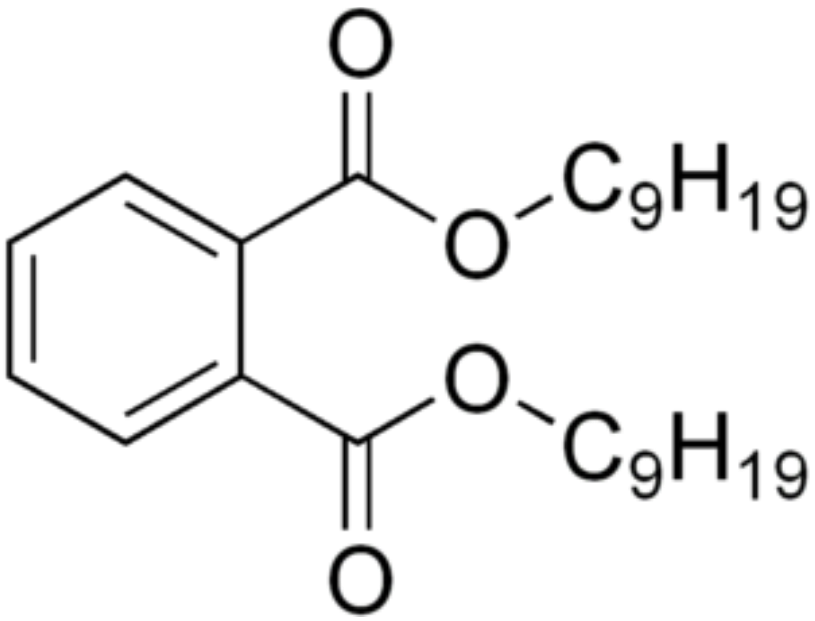
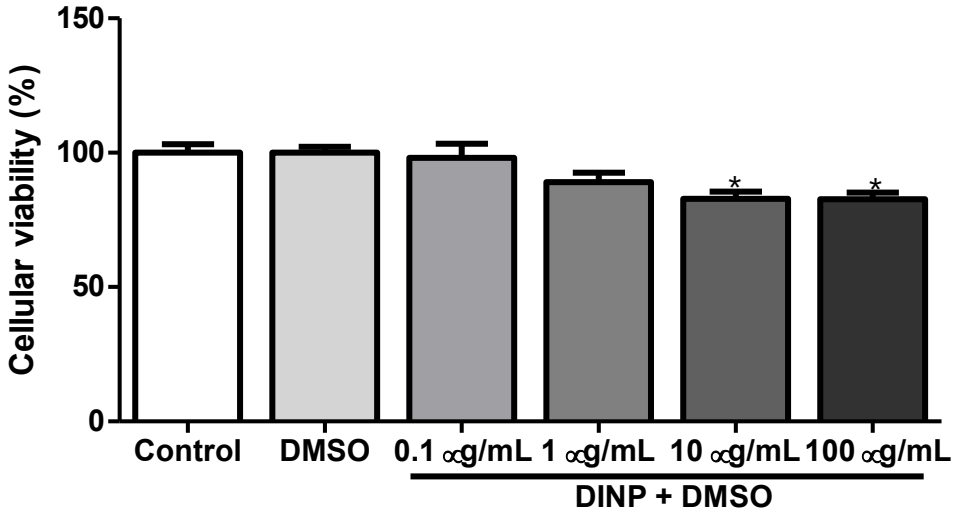


Fig. 1

A)


150
100
50
0
Cellular viability (%)
*
*
Control
DMSO
0.1 ∝g/mL
1 ∝g/mL
10 ∝g/mL
100 ∝g/mL
DINP + DMSO

B)

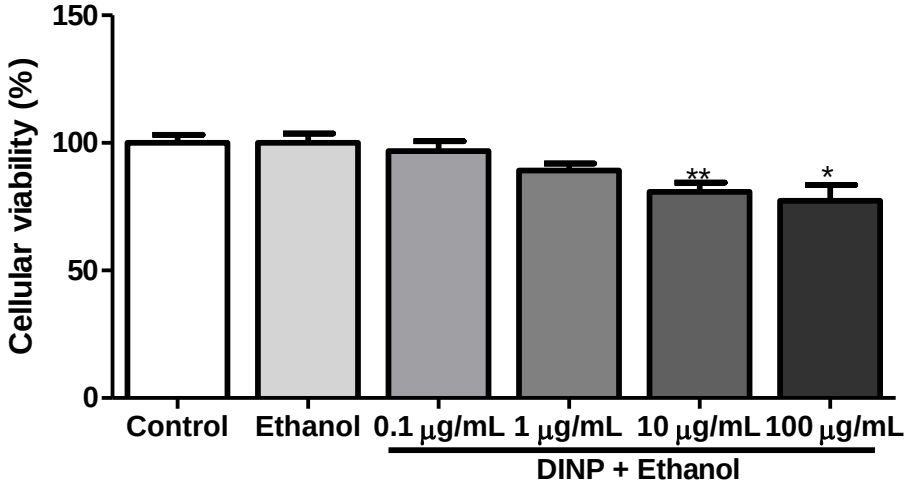

150
100
50
0
Cellular viability (%)
**
*
Control
Ethanol
0.1 μg/mL
1 μg/mL
10 μg/mL
100 μg/mL
DINP + Ethanol

Fig. 2

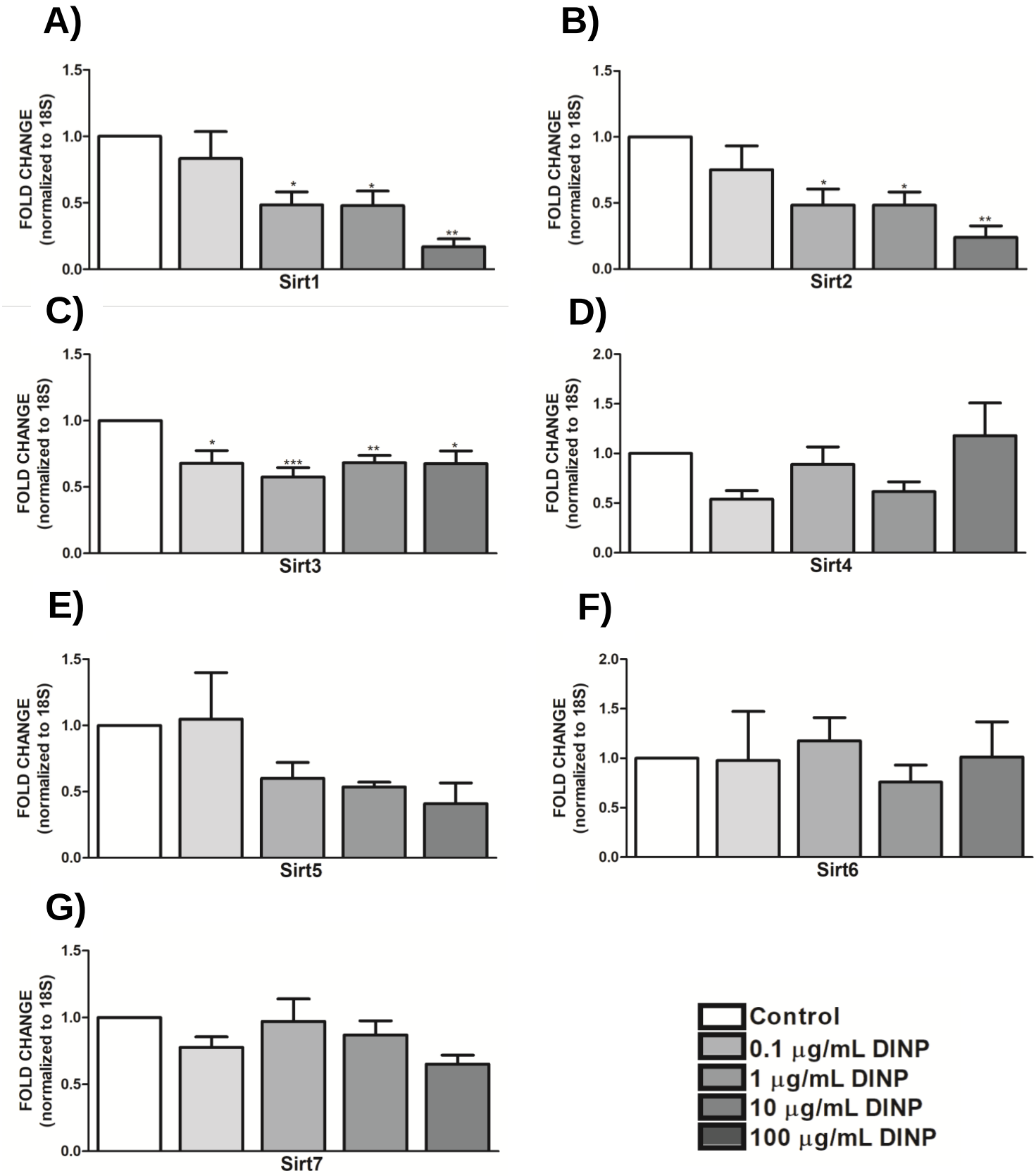

A)
B)
C)
D)
E)
F)
G)
FOLD CHANGE (normalized to 18S)
Sirt1
Sirt2
Sirt3
Sirt4
Sirt5
Sirt6
Sirt7
Control
0.1 μg/mL DINP
1 μg/mL DINP
10 μg/mL DINP
100 μg/mL DINP

Fig. 3

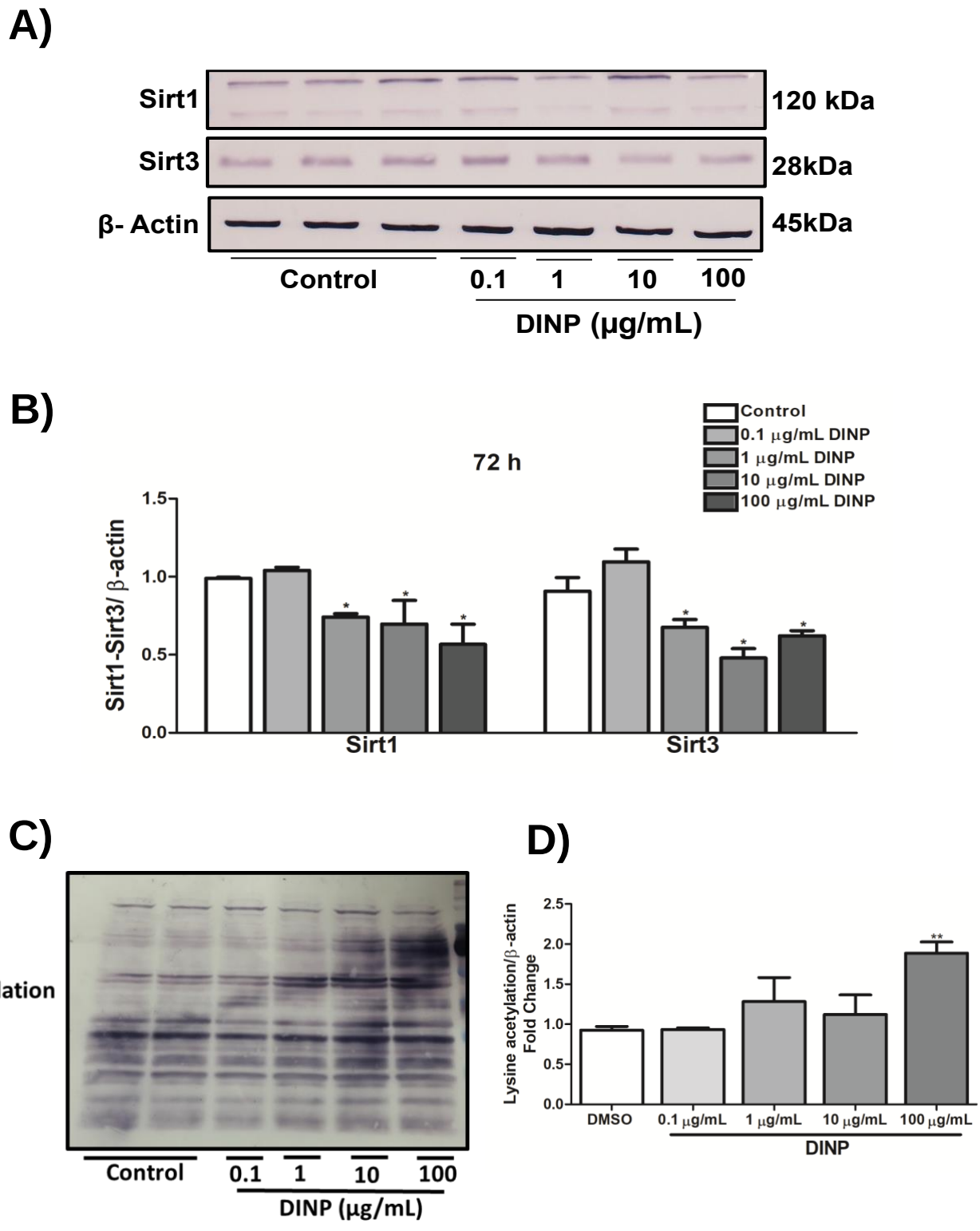

A)
Sirt1
120 kDa
Sirt3
28kDa
β- Actin
45kDa
Control
0.1
1
10
100
DINP (µg/mL)
B)
Control
0.1 µg/mL DINP
1 µg/mL DINP
10 µg/mL DINP
100 µg/mL DINP
72 h
Sirt1-Sirt3/ β-actin
1.5
1.0
0.5
0.0
Sirt1
Sirt3
C)
Acetylation
Control
0.1
1
10
100
DINP (µg/mL)
D)
Lysine acetylation/β-actin
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
DINP

Fig. 4

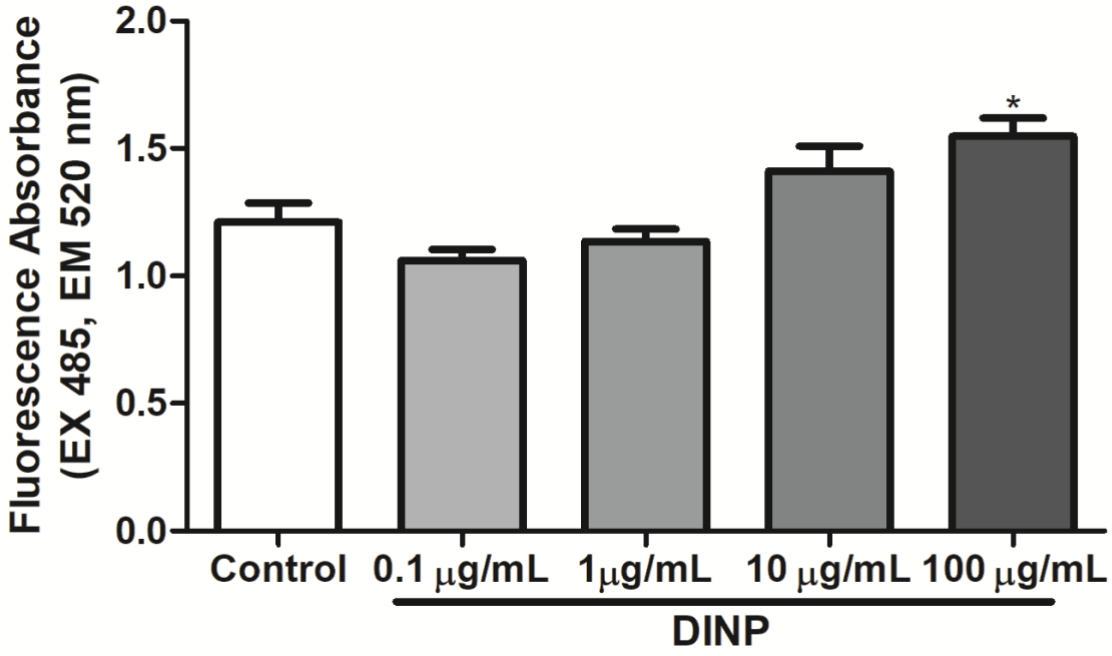

Fluorescence Absorbance
(EX 485, EM 520 nm)
2.0
1.5
1.0
0.5
0.0
*
Control
0.1 μg/mL
1μg/mL
10 μg/mL
100 μg/mL
DINP

Fig. 5

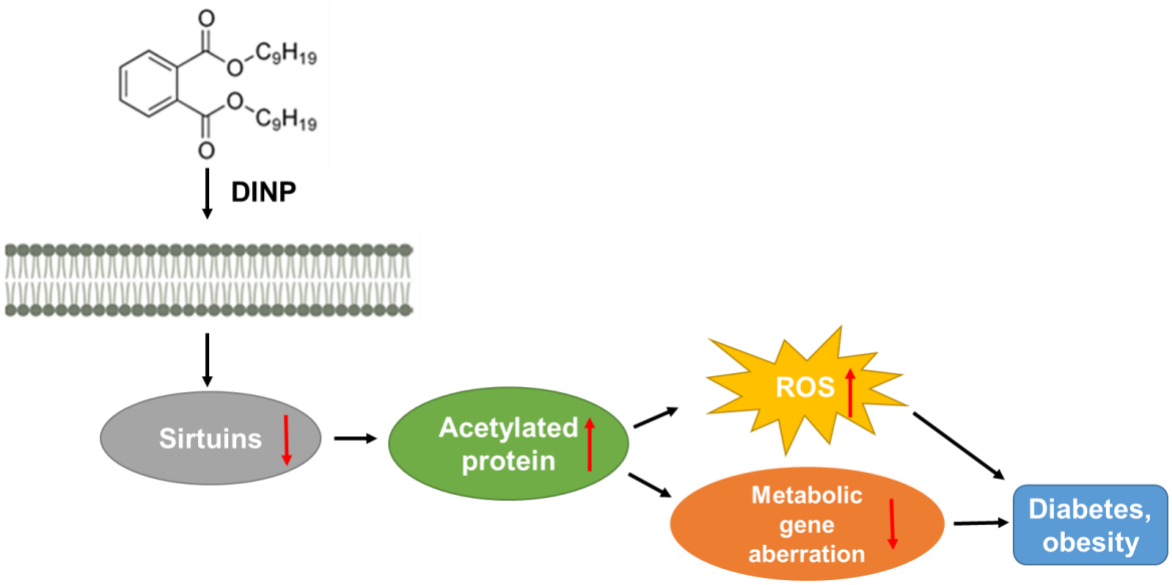

O
O
C_9H_{19}
O
C_9H_{19}
O
DINP
Sirtuins
Acetylated protein
ROS
Metabolic gene aberration
Diabetes, obesity

Fig. 6