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Alpha-lipoic acid represses IL-1B and IL-6 through DNA methylation in ovarian cells

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ABSTRACT

Alpha-lipoic acid (ALA) is an endogenous molecule with pleiotropic effects, including antioxidant and antiinflammatory activity mediated by NF-kB nuclear factor, that has the potential to regulate pro- and anti-inflammatory cytokines. ALA-induced downregulation of two major pro-inflammatory cytokines, IL-1B and IL-6, has been ascertained in different tissues and experimental models.

In pregnant women, ALA-dependent modulation of inflammation is beneficial for the implantation and the management of threatened miscarriage and preterm delivery.

Recent data indicate that *IL-1B* and *IL-6* expression are modulated by DNA methylation; we previously demonstrated that hypomethylation of these two genes is associated to increased expression in human brain.

We then investigated whether ALA-dependent *IL-1B* and *IL-6* downregulation is mediated by epigenetic changes in ovarian experimental models, studying the DNA methylation profile of *IL-1B* and *IL-6* 5′-flanking regions in human ovarian epithelial cell lines.

We found that ALA induce hypermethylation of *IL-1B* and *IL-6* 5′-flanking regions and is associated to IL-1B and IL-6 mRNA and protein downregulation.

These data provide novel insights on the antiinflammatory effects of ALA and support its clinical use in pregnancy complications.

1. Introduction

Alpha-lipoic acid (ALA) is an endogenous molecule also known as thioctic acid; it is known as a coenzyme in the Krebs cycle and the primary role of this molecule is to assist in acyl-group transfer in biochemical reactions [1].

In the clinical setting ALA is largely used for its recognized, powerful antioxidant effect, because it can counteract free radicals in both lipophilic and hydrophilic milieu and since the antioxidant properties are preserved when ALA is both in the oxidized and reduced form [2–4].

Another relevant property that captures the attention on ALA as a molecule of potential clinical interest is its strong antiinflammatory effect [5]. It is well known that this effect is mediated by modulation of nuclear factor kB (NF-kB) which, in turn, has the potential to regulate the production of pro- and anti-inflammatory cytokine [6]. IL-1B and IL-6 are two main pro-inflammatory cytokines under the transcriptional control of NF-kB in different tissues [7–11]. It was recently demonstrated that both IL-1B and IL-6 [12] as well as other pro-inflammatory cytokines are regulated by ALA mostly through modulation of NF-kB activity [13–17].

Therefore, ALA has been proposed as useful adjuvant treatment in the management of different human diseases including diabetes, cardiovascular disease, bone loss, inflammatory chronic disease, neurodegeneration, peripheral neuropathies and other diseases [18]. In the last fifteen years, ALA has been also suggested and studied for its positive effects on pregnancy complications and perinatal defects [19]. On the offspring, ALA demonstrated to be effective in preventing neural tube defects in fetuses [20] and in contrasting fetal membrane weakening [21]. On the mothers, ALA showed positive effects against pregnancy-associated peripheral neuropaties [22] and in contrasting gestational diabetes-associated complications [23] thereby reducing the risk of pregnancy loss [24]. In pilot studies, ALA supplementation showed positive effects in restoring subchorionic hematoma in women

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with threatened miscarriage both in association with vaginal progesterone and alone [25,26]. Recently, vaginal use of ALA has been investigated as a new therapeutic approach useful to counteract the progressive cervical shortening after primary tocolysis in women at risk of preterm delivery [27]. All these beneficial effects result in a generalized ALA capacity in reducing the risk of miscarriage and pregnancy loss due to different clinical and physiological conditions [28].

Recent findings evidenced that epigenetic mechanisms regulate IL-1B and IL-6, among other pro-inflammatory cytokines. Specifically, these genes appear to be under the control of differential DNA methylation in the promoters regions [29–31]. Our laboratory contributed to this field reporting very recently that these two genes are modulated by DNA methylation in human brain during specific pathological conditions. *IL-1B* promoter was specifically hypo-methylated in the brains of patients with Tuberous Sclerosis Complex (TSC), both in tuberal and perituberal tissues compared to control samples, and the hypo-methylation was positively associated to *IL-1B* mRNA over-expression [32]. Moreover, we established that both *IL-1B* and *IL-6* are modulated by DNA methylation in brain tissue from autopsy of Alzheimer's Disease patients at different stages of the pathology [33].

These recent findings allowed us to hypothesize that the mechanisms by which *IL-1B* and *IL-6* are regulated by ALA supplementation and NF-kB modulation, could involve some change in the DNA methylation profile of the respective gene promoters in ovarian experimental models. We therefore decided to analyze the DNA methylation profile of *IL-1B* and *IL-6* 5′-flanking regions in two cancer (SKOV3 and TOV-21G) and one normal (IOSE-398) human ovarian epithelial cell lines, focusing both on the gene expression and to the secretion of the two cytokines. The analysis of the methylation profile was performed by bisulphite assay, according to our recent protocol that takes advantage of the unbiased efficiency of non-CpG-Methylation Insensitive Primers (MIPs), allowing to assess both CpG and non-CpG DNA methylation at single cytosine resolution [34].

2. Materials and methods

2.1. Cell cultures

The human ovarian epithelial cancer cell lines SKOV3 and TOV-21G were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). SKOV3 were maintained in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FCS, Sigma-Aldrich Corp., St. Louis, MO), TOV-21G were maintained in Medium 199:MCDB 131 (Sigma-Aldrich Corp.) supplemented with 15% FCS. The non-tumorigenic, ovarian epithelial, SV40 Tag-immortalized OSE-derived lines, IOSE 398-398, were cultured in medium 199:MCDB 105 (Sigma-Aldrich Corp.) containing 5% FCS. All the media were supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/mL). Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2-95% air. The IOSE 398-398 cell line was generously provided by Dr. D. Huntsman (Department of Pathology and Laboratory Medicine and Department of Molecular Oncology, British Columbia Cancer Agency University of British Columbia, Vancouver, Canada).

According to the experimental plan, cells were plated in the appropriate medium and, after 24 h of growth, were shifted to control (10% FCS, DMSO) or ALA 0.5 mM (dissolved in DMSO at a final 10% concentration) supplemented medium. After 24 h of culture, cells were collected and stored at −80 °C for the following DNA or RNA purification. At 24 h surnatants were also collected and stored at −80 °C for the following ELISA test. Experiments were repeated at least three times.

2.2. DNA methylation study by bisulphite modification and genomic sequencing

DNA was extracted from tissues using the DNeasy Blood and Tissue Kit (Cat. #69504, Lot #136269927) and the Qiacube instrument (Qiagen, Milan, Italy).

Bisulphite analysis of *IL-1B* and *IL-6* promoter methylation was performed as previously described [32] using the EpiTect Bisulphite kit; 50 ng of bisulphite-treated genomic DNA was amplified using specific PCR primers [33] and PCR products obtained were cloned using the PCR Plus Cloning Kit (both from Qiagen, Milan, Itay). After white/blue screening on LB-agar plates, at least ten positive clones were analyzed per experimental condition using M13 primers for sequencing. Sequencing reactions of purified plasmid DNA were performed. Clones were sequenced by the cycle sequencing method (in service by PRIMM, Milan, Italy).

Modified cytosine residues were recognized through comparison with the original DNA sequence and methylation status of each single cytosine in every sequenced clone and reported as 1/0 value in an Excel spreadsheet (methylated: 1; unmethylated: 0). For each experimental sample we obtained the methylation percentage of each single cytosine by calculating the number of methylated cytosines divided by the number of clones sequenced per 100 (n° methylC/n° sequenced clones ×100).

We then calculated the average methylation percentage over the tissue samples for each experimental condition.

GenBank accession numbers, primer names, sequences and position and expected products of the Methylation Insensitive Primers (MIPs) used for bisulphite analysis are presented in Table II. These primers allowed assessing methylation status of plus (5′- > 3′) DNA strand.

We also used different bisulphite modification assays [34] as random control in samples characterized by low and high (CpG and non-CpG) methylation to ensure that cytosine conversion was complete. In all these cases, the observed methylation patterns were similar. As negative controls of bisulphite modifications we used unmethylated purified PCR products of gene promoters, obtained from genomic DNA as template with the same MIPs primers used for bisulphite PCR; these purified PCR products where also methylated *in vitro* with SssI methylase (New England Biolabs), that methylates only cytosines in CpG dinucleotides to be used as positive controls.

2.3. mRNA expression study by real-time PCR

RNA from cultured cells was extracted with the RNeasy mini kit (Qiagen, Milano Italy); 1 μg of total RNA was used for cDNA synthesis and 1 μg of total cDNA was used for each real-time reaction; analyses were performed in triplicate for each sample as previously described [15]. Total cDNA levels were standardized by normalizing them to the B-actin control and presented as the fold increase (ratio of the experimental gene value/B − actin gene value) over the control sample. GAPDH and 18S were also used to normalize the PCR reactions with comparable results (not shown)

2.4. Cytokines production study by ELISA test

Levels of cytokines released in the culture medium were determined by ELISA following the manufacturer's instructions, using the following kits: IL-1B beta, sensitivity: 6.5 pg/mL (Abcam, ab46052); IL-6, sensitivity: 0.7 pg/mL (Quantikine, R & D, D6050). ELISA plates were analyzed by a microtiter plate reader (Opsys MR; Dinex Technologies).

2.5. Statistical analysis

Statview 5.0 (SAS Institute, Milan, Italy) statistical software was used. Mann-Whitney non-parametric tests were applied for the analysis of the methylation data since the experimental method (sequencing of at least 10 clones for each experimental replicate) results in percent values (methylation%) for many cytosines (non-correlated values) in each sample. One-way ANOVA was computed and Tuckey's post-test was used to calculate any significant difference in Real-Time PCR and in