行政院國家科學委員會專題研究計畫 成果報告

天然物對砷所誘發之血管粥狀硬化抑制機制之探討
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-041-010-
執 行 期 間 ：95 年 08 月 01 日至 96 年 10 月 31 日
執 行 單 位 ：嘉南藥理科技大學生物科技系(所)

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 97 年 01 月 30 日

中文摘要

砷是本省主要的環境污染源之一，近年來的研究指出砷是誘發血管病變，癌症及免疫毒性的危險因子。由於巨噬細胞在先天性免疫及引發動脈粥狀硬化上扮演重要角色，研究顯示人類單核球受砷影響，會造成的分化不全及基因表達的改變。

本研究主要探討天然含硫抗氧化劑-硫辛酸對砷所引起與血管粥狀硬化相關的分子的表達的影響。目前的結果顯示低濃度的砷(≦2.5 μM)作用 24 h，對 THP-1 衍生的巨噬細胞並無明顯的細胞毒性；但是卻能顯著增加清道夫受體-CD36 的表面蛋白的表現；砷(1 μM)也會顯著增加細胞對 DiI-oxLDL 的攝入。同時加入硫辛酸(50 μM)則可顯著減低 CD36 的表面蛋白的表現並減少 DiI-oxLDL 的攝入，因此硫辛酸可能具有預防砷引起動脈粥狀硬化的功效。

關鍵字： 砷、硫辛酸、CD36、oxLDL

Abstract

Arsenic is considered a potent human hazard because of its neoplastic outcomes. However, increasing epidemiological evidence suggests that there is also link between arsenic exposure and risk of vascular diseases related to atherosclerosis. The effect of arsenic on markers and marker gene expression, however, depends on cell type, arsenical species and dose.

The aim of the project is to investigate the effects and mechanisms of natural antioxidant, α-lipoic acid (LA), on arsenic-induced molecular and cellular events related atherogenesis. The class B scavenger receptor, CD36, binds to oxLDL, is present in atherosclerotic lesions. Exposure of THP-1 human monocyte-derived macrophages with arsenic (<2.5 μM) for 24 h did not cause significant cytotoxicity, while markedly elevated CD36 cell surface protein expression. Cotreatment of cells with LA (50 μM) successfully attenuated CD36 surface protein expression. Coincidently, As induced the uptake of 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanide perchlorate (DiI)-labeled oxLDL uptake in THP-1-derived macrophages and cotreatment of LA markedly decreased oxLDL uptake dose-dependently. Current result implies that LA may play a role in ameliorating arsenic-induced atherosclerosis.

Keywords: arsenic, α-lipolic acid, CD36, oxLDL

Introduction

The primary cause of heart disease and stroke is atherosclerosis, a disease characterized by an accumulation of lipid laden foam cells beneath the aortic endothelium. There is considerable evidence indicated that atherogenesis is initiated and promoted by lipid oxidation of LDL, ultimately leading to oxidative modification of apolipoprotein B. Exposure of monocytes to oxLDL may alter gene expression and signaling making them more susceptible to pro-atherogenic stimuli. The migration of monocytes into the intima and the conversion of monocytes/macrophages into foam cells represent initial steps in atherosclerosis. Current strategies to prevent atherosclerosis are aimed either at lowering the cholesterol load of lipoproteins or at reducing oxidative stress [1].

Scavenger receptors (SRs) are thought to play a significant role in atherosclerotic foam cell development because of their ability to bind and internalize modified lipids [2]. SR class B (SR-B), has been identified as the major oxLDL receptor. CD36, a member of the SR-B family, plays a quantitatively significant role in oxLDL binding to macrophages [3]. CD36 is highly expressed on lipid-laden macrophages in human atherosclerotic aorta [4], possibly as a result of a positive feedback loop mediated by oxLDL and its lipid content [5, 6]. Inhibition of CD36 expression has been demonstrated to reduce the development of atherosclerosis in atherosclerosis-prone apolipoprotein E knockout mice [7]. These findings suggest that CD36 plays a pro-atherogenic role in foam cell formation.

At the transcriptional level, CD36 expression is induced by oxLDL via the peroxisome proliferator receptor gamma (PPARγ) and the NF-E2-related factor (Nrf2) [8-10]. PPARγ is a member of the nuclear hormone receptor superfamily. PPARγ heterodimerizes with the retinoid X receptor (RXR) and functions as a transcriptional regulator of genes that modulate lipid metabolism and adipocyte gene expression [11].

Environmental factors are considered key determinants of cardiovascular disease. Many epidemiological studies report that exposure to arsenic linked to increased incidence of cardiovascular disease, and cancers of skin, bladder, lung, and, possibly, of other internal organs [12, 13]. One of the most significant manifestations of arsenic toxicity is blackfoot disease, which has been attributed to arteriosclerosis obliterans and thromboangiitis obliterans caused by peripheral artery disease (PAD) and leads to spontaneous loss of extremities [14]. The mechanisms by which arsenic causes vascular disease are not well known, but animal studies suggest that the chemical propensity of arsenic to oxidize vicinal thiols could potentially affect a number of cellular proteins with reactive thiols [15, 16] including endothelial NO synthase (eNOS) [17]. Arsenic also induces oxidant stress and NF-kappa B activation in cultured aortic endothelial cells [15].

In addition to endothelial cells, monocytes/macrophages constitute major targets for As toxicity [18]. Low concentration of As (1 μM) inhibited monocytes differentiated into macrophages [19] ; on the other hand, higher concentration of As (> 4 μM) inhibited NF-κB activation and induced apoptosis [20].

The oxidative hypothesis of atherosclerosis development has attracted extensive investigation of a possible preventive role of antioxidants [21]. Dietary antioxidants, such as vitamin E or polyphenolic flavonoids, as well as polyphenol-rich foods, not only protect low-density lipoprotein (LDL) from oxidation but also reduce the development of atherosclerotic lesions [22-25].

Alpha-lipoic acid (LA), also known as thioctic acid, is a naturally occurring antioxidant compound that is synthesized in small amounts by plants and animals, including humans [26]. Endogenously synthesized LA is covalently bound to specific proteins, which function as cofactors for several important mitochondrial enzyme complexes. There is increasing scientific and medical interest in potential therapeutic uses of pharmacological doses of free LA [27].

Recent animal experiment demonstrated that dietary LA is a promising protective agent for reducing cardiovascular complications of diabetes [28].

It is well-known that monocyte-derived macrophages are critical cells that are present in all stages of atherogenesis, however, most current researches focused only on the apoptotic effects of As in this type of cells. There is a scarcity of data examining the roles of As and LA in the uptake of oxLDL or the expression of CD36 in human monocyte-derived macrophages. Thus, the aim of this study is to determine the effects of As and LA on the expression and function of CD36 in THP-1-derived macrophages.

Materials and Methods

Cell culture. The monocyte-like cell line THP-1 was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The THP-1 cells were cultured in RPMI 1640 medium, which contained 2 mM L-glutamine, 4.5 g/L glucose, 15 mM HEPES (Sigma), 1.0 mM sodium pyruvate, 15 % fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37^{o}C in 5% CO_2 incubator.

Differentiation into macrophages was achieved by treating the THP-1 cells in 6-well plates ($1x10^5$ cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 nM) for 72 h. Differentiated THP-1 cells were incubated at 37 ^{o}C with sodium arsenite ($NaAsO_2$) (Sigma, 0, 1, 2.5 μM) alone or co-incubated with indicated concentration of α-lipolic acid (Sigma) for 24 h. The cells were then subjected to cell viability, CD36 cell surface protein, CD36 and PPARγ mRNA expression and DiI-oxLDL uptake analyses as described below.

Cell viability analysis. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan [29]. Cells were incubated with MTT (0.5%) for 4 h at 37 ^{o}C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantitated by measurement of the absorbance at 550 nm.

CD36 cell surface protein expression. Treated differentiated THP-1 cells were washed with and scraped in PBS, pH 8.2. The pellet was incubated with fluorescein (FITC)-conjugated murine anti-human CD36 (clone FA6.152, Immunotech, Beckman Coulter) or isotype controls on ice for 60 min before washing twice with PBS (250 x g, 5 min). The cells were investigated in duplicates by flow cytometry (Coulter EPICS XL, Beckman Coulter). Data were acquired from 15,000 cells (events), and the fluorescent intensity of CD36 expression was determined and expressed as the mean fluorescence intensity (MFI).

RNA extraction. Total cellular RNA was prepared using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK) from treated differentiated THP-1 cells. Isolated RNA was quantified by RiboGreen RNA Quantitation Kit (Invitrogen) in FLx800 Multi-Dectection Microplate Reader (BioTek, Winooski, VT, USA).

CD36 and PPARγ gene expression analysis using quantitative real-time RT-PCR. Reverse-transcription was carried out using 1 μg RNA and High-Capacity cDNA Archive kit

(Applied Biosystems, Foster City, CA, USA) followed by PCR in real-time using Hot Start TaqMan Master Mix Reagent Kit (Applied Biosystem) on iQ5 Real time PCR detection system (BioRad, Hercules, CA, USA). The reactions were performed using the following cycling conditions for CD36: 95^{o}C for 10 min, then 40 cycles of 95 ^{o}C for 45 sec, 60 ^{o}C for 45 sec and 72 ^{o}C for 1 min. The sequences of the primers and probe of CD36 used were as follows: forward primer, 5'-CTGAGGACAACACAGTCTCTTTCC-3'; reverse primer, 5'-ACTGTGAAGTTGTCAGCCTCTGTTC-3'; probe, 5'-6FAM-TGGTGCCATCTTCGAACCTTCACTATCAG-TAMRA-3' [33]. The reactions were performed using the following cycling conditions for PPARγ: 95^{o}C for 10 min, then 40 cycles of 95 ^{o}C for 45 sec, 52 ^{o}C for 45 sec and 72 ^{o}C for 1 min. The sequences of the primers and probe of PPARγ used were as follows: forward primer, 5'-CATGGCAATTGAATGTCGTG-3'; reverse primer, 5'-TTCTCCGGAAGAAACCCTTG-3'; probe, 5'-6FAM-AAGCTTCTGGATTTCACTATGGAGTTCAT-TAMRA-3'. PCR results were then normalized to the expression of GAPDH in the same samples. Primers and probe for GAPDH were from TaqMan GAPDH control reagent (Applied Biosystems)**.**

Preparation and oxidation of LDL. LDL (d 1.019–1.063) was prepared from the plasma of healthy donors by sequential ultracentrifugation [30]. Lipoprotein was desalted and concentrated by filtration (Centricon 4, Amicon, Beverly, MA) against PBS at 450 x g, 4^{o}C for 120 min. The protein concentration was measured by the method of Bradford [31], using bovine serum albumin as a standard.

Preparation of DiI-oxLDL. For preparation of DiI-LDL, 150 μL LDL (10 mg/mL), 50 μL DiI (3 mg/ml in DMSO) and 800 μL PBS were mixed and incubated at 37^{o}C in dark for 16 h. DiI-LDL was then incubated with 5 μM Cu^{2+} in PBS at 37^{o}C overnight in dark for 18 h. Unbound dye and copper ions were removed through ultrafiltration (Centricon 4, Amicon, Beverly, MA) against ddH_2O-EDTA (0.24 mM) at 8000 x g, 4^{o}C for 120 min.

DiI-oxLDL uptake by macrophages. Treated differentiated THP-1 cells were then incubated with DiI-oxLDL (50 μg/ml) for another 24 h. The cells were washed and then investigated in duplicates by flow cytometry. Data were acquired from 15,000 cells (events), and the DiI-oxLDL uptake was determined and expressed as the mean fluorescence intensity (MFI).

Statistical analyses. *t*-tests were used to assess significant differences in parameters measured in the presence and absence of test substances and the level of significance was set at $p<0.05$. All experiments have been performed at least three times.

Results

Effect of As on the viability of THP-1 macrophages

THP-1 cells were traditionally used as a model monocyte/macrophage cell line. These cells can be induced to differentiate into macrophages by a variety of stimuli including phorbol 12-myristate 13-acetate (PMA). It has been demonstrated that As is a potent oxidant and induced apoptosisin monocytes/macrophages. **Figure 1** shows that THP-1 macrophages treated with 5 μM As for 24 h caused more than 50% of cell death. On the other hand, lower concentrations of As (1

and 2.5 μM) did not exert significant cytotoxicity as detected by MTT analysis. To test the effects of As and LA on gene expression, the rest of experiments were performed under nontoxic condition.

Effect of As and LA on CD36 surface protein expression of THP-1 macrophages

THP-1 cells were widely used as a model for investigating regulation of CD36 expression on cells of the monocyte/macrophage lineage. THP-1 monocytes showed little surface expression of CD36, while PMA-treated THP-1 cells increased in surface CD36 expression that correlated with differentiation process. **Figure 2** shows the effects of As and LA on CD36 cell surface protein expression, which was measured as geometric mean of fluorescent intensity (MFI), in PMA-differentiated THP-1 cells. It is demonstrated that As (1 and 2.5 μM) can significantly induced surface CD36 protein expression in THP-1-derived macrophages ($p<0.05$). The As-induced CD36 surface protein expression can be markedly attenuated by 50 μM LA ($p<0.05$ and 0.01, for 1 and 2.5 μM As, respectively).

Effect of As and LA on CD36 and PPARγ mRNA expression

To further investigate whether As and LA regulate CD36 gene expression in the transcription level, RT-Q-PCR was employed as described in Materials and Methods. **Figure 3** shows that treatment of THP-1 macrophages with LA (75 μM) alone increased CD36 mRNA expression by about 50~60% as compared with control ($p<0.01$). On the other hand, 2.5 μM As significantly reduced CD36 mRNA expression by ~28% as compared with control ($p<0.05$). The As-repressed CD36 can be completely reversed by co-treatment of cells with 2.5 μM As and LA (50 and 75 μM) and the CD36 mRNA expression increased by more than two folds as compared with As alone ($p<0.05$ and 0.01, respectively).

Since CD36 expression is regulated by PPARγ activation at transcription level, we further examined the effects of As and LA on PPARγ mRNA expression. **Figure 4** reveals that THP-1 macrophages treated with LA (50-75 μM) alone did not significantly affect PPARγ expression while 10 μM LA slightly increased as compared with control ($p<0.05$). However, treatment of cells with 2.5 μM As alone markedly diminish PPARγ mRNA expression by more than 70% as compared with control ($p<0.01$). The As-caused PPARγ repression can be completely relieved by co-treatment of cells with 2.5 μM As and LA (50 and 75 μM). These results imply that As suppressed CD36 expression through down-regulation of PPARγ expression and addition of LA (50 and 75 μM) can overturn this effect.

Effect of As and LA on DiI-oxLDL uptake by THP-1 macrophages

The above results led us to examine the effects of LA on cellular uptake of oxLDL. DiI-oxLDL is known to be bound to and/or taken up via CD36. THP-1-derived macrophages were pretreated with As and LA for 24 h. Next, the cells were exposed to DiI-oxLDL for 24 h at 37^{o}C. **Figure 5A** is the representative histogram and demonstrates that co-treament of LA (50 μM) significantly lower the DiI-oxLDL uptake in As (1μM)-treated THP-1 macrophages. **Figure 5B** summaries the effect of As and LA and demonstrates that pre-treatment of THP-1-derived

macrophages with As (1 μM) for 24 h increased DiI-oxLDL uptake (p<0.05). Co-treatment of these cells with LA (10 and 50 μM) significantly reduced As-stimulated oxLDL uptake to the level compatible to or lower than normal cells.

Discussion

The formation of foam cells in the intima, the fatty streak lesion, is an important early event in atherogenesis [32]. CD36, a class B scavenger receptor, is thought to bind and internalized oxLDL during foam cell formation. To better understand the mechanisms by which arsenic causes atherosclerosis and possible beneficial effect of α-lipolic acid, we used the monocytic leukaemia cell line THP-1, which differentiates to macrophages in response to phorbol 12-myristate 13-acetate (PMA), to investigate their effects of on the expression and activity of CD36 in human monocyte/macrophages.

PMA, a protein kinase C (PKC) activator, is a potent inducer of CD36 [33]. It has been shown that CD36 is upregulated by oxLDL via the PPARγ pathway [8] and via PKC [34]. In this study, we found that low concentrations of As (1 and 2.5 μM) which would not cause significant cytotoxicity, but significantly elevated CD36 surface protein expression in PMA-activated THP-1 cells by about 10-15% (**Figure 2**). RT-Q-PCR surprisingly revealed that As (2.5 μM) suppressed CD36 and PPARγ mRNA expression by about 28% and 72%, respectively (**Figures 3 and 4**). Current data implied that As may exert completely opposite effects on the upstream transcription factor PPARγ and CD36 mRNA expression vs. downstream CD36 surface protein expression. However, more experiments are needed to clarify the effect of As on the storage of preformed CD36 protein in intracellular pools of cells and receptor recycling [35].

It has been demonstrated that long-term exposure of macrophages with As (1 μM) significantly decreased the expression of integrins, CD11b and CD29, as well as macrophagic differentiation marker, CD71, detected by flow cytometry [18]. The same authors also demonstrated that As (≧ 4 μM) inhibited NF-κB activation in macrophages [20]. PPARγ activation is well-known down-regulating NF-κB activation. Current result suggests that low concentration As decreases PPARγ mRNA expression and may also down-regulate PPARγ activation, which in turn induces NF-κB activation and inflammatory response.

Alpha-lipoic acid (LA), a potent antioxidant, shows strong inhibition against As-induced CD36 cell surface protein expression and dose-dependently reduced DiI-oxLDL uptake. This result indicates that LA can prevent As-induced foam cell formation. However, mechanism on gene expression or upstream transcription factor involved remains to be solved.

計畫自評

本研究原計畫為三年期，但僅通過第一年的補助。目前已完成砷對THP-1巨噬細胞的毒性、CD36表面蛋白表達及DiI-oxLDL攝入的分析。目前正致力將相關機轉，包括PPARγ-CD36 mRNA表達的研究，以準備投稿。

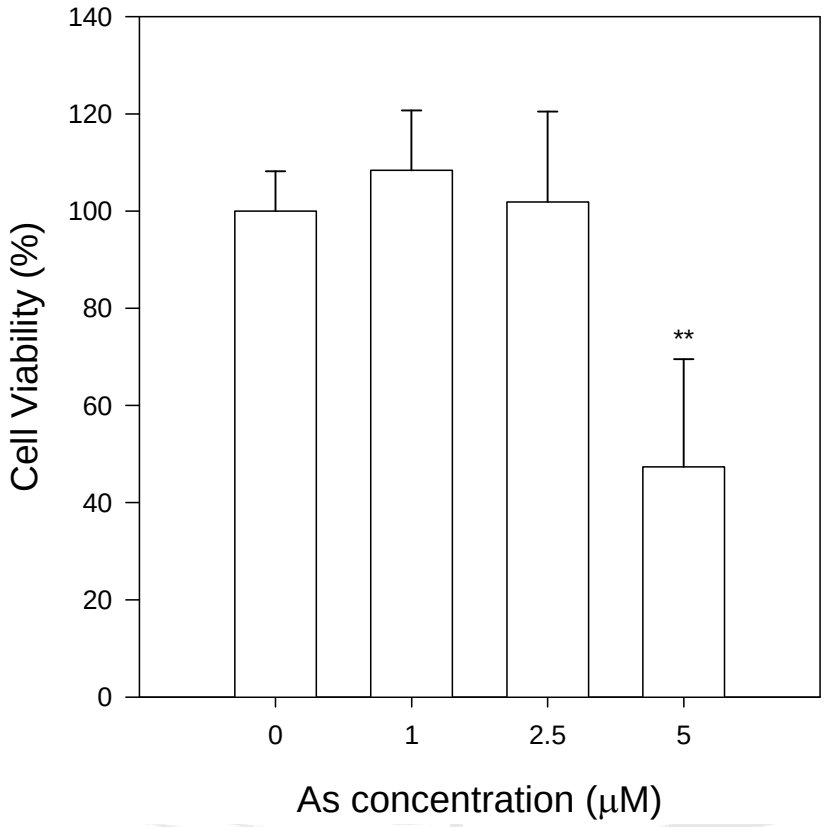


Figure 1. Effect of arsenic concentration on cell viability of THP-1 macrophages. Differentiation into macrophages was achieved by treating the THP-1 cells in 6-well plates ($1x10^5$ cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 nM) for 72 h. Differentiated THP-1 cells were incubated at 37 ^oC with sodium arsenite ($NaAsO_2$) (Sigma, 0, 1, 2.5 μM) alone for 24h. The cell viability was analyzed using MTT as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. *, $p<0.05$ represents significant differences compared with normal control.

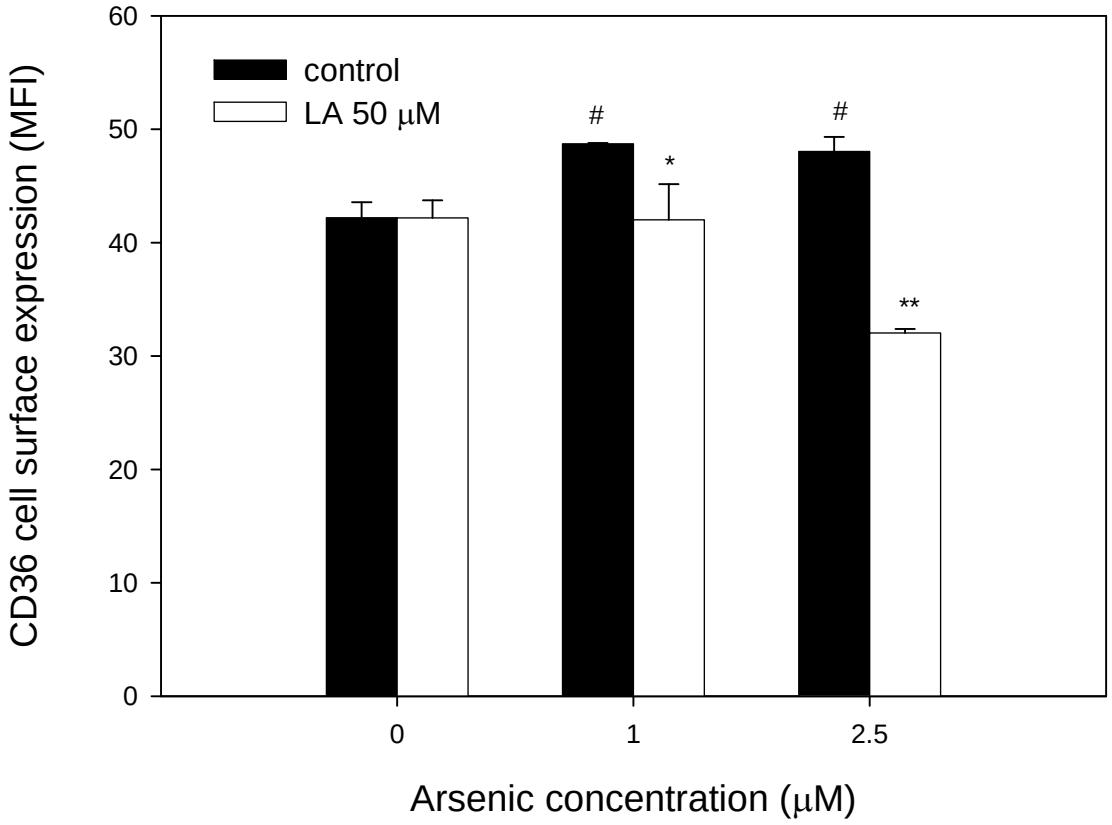


Figure 2. Effects of arsenic and α-lipolic acid (LA) on CD36 cell surface protein expression in THP-1 macrophages. Differentiation into macrophages was achieved by treating the THP-1 cells in 6-well plates ($1x10^5$ cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 nM) for 72 h. Differentiated THP-1 cells were incubated at 37 ^oC with sodium arsenite ($NaAsO_2$) (0, 1, 2.5 μM) alone or in combination with LA (50 μM) for 24h. CD36 cell surface protein expression was analyzed using flow cytometry as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. #, $p<0.05$ represents significant differences *vs.* normal control group. *, $p<0.05$; **, $p<0.01$ represent significant differences *vs.* cells treated with the same concentration of As alone.

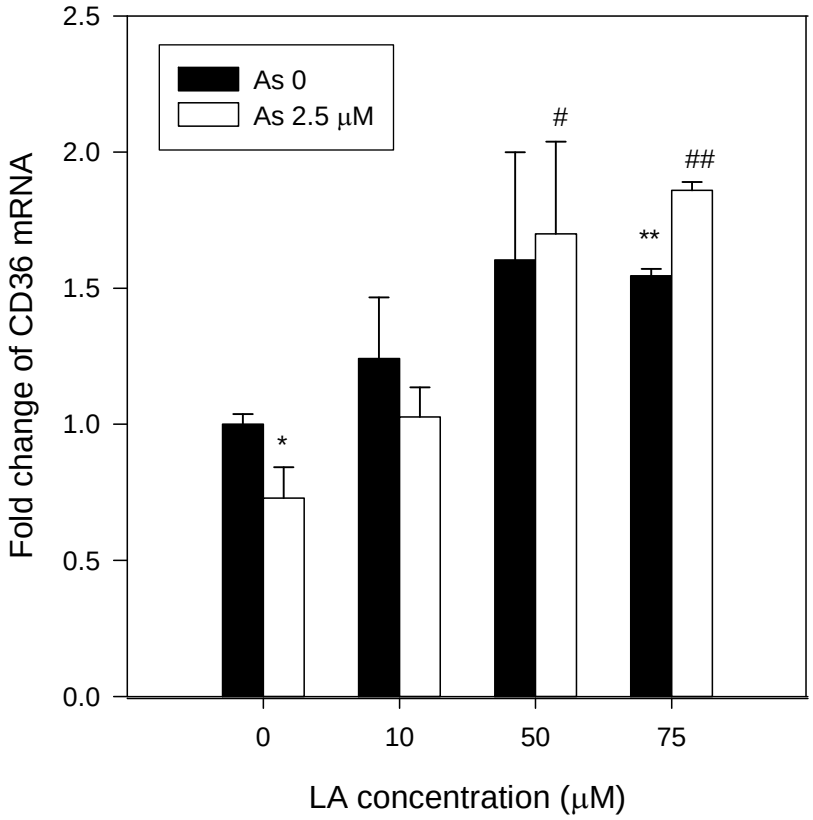


Figure 3. Effects of As and LA on CD36 mRNA expression in PMA-differentiated THP-1 cells. Differentiation into macrophages was achieved by treating the THP-1 cells in 6-well plates ($1x10^5$ cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 nM) for 72 h. Differentiated THP-1 cells were incubated at 37 ^oC with sodium arsenite ($NaAsO_2$) (0 and 2.5 μM) alone or in combination with LA (10, 50 and 75 μM) for 24h. CD36 mRNA expression was analyzed using RT-Q-PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. #, $p<0.05$; ##, $p<0.01$ represent significant differences *vs.* cells treated with the same concentration of As alone. *, $p<0.05$; **, $p<0.01$ represent significant differences *vs.* normal control.

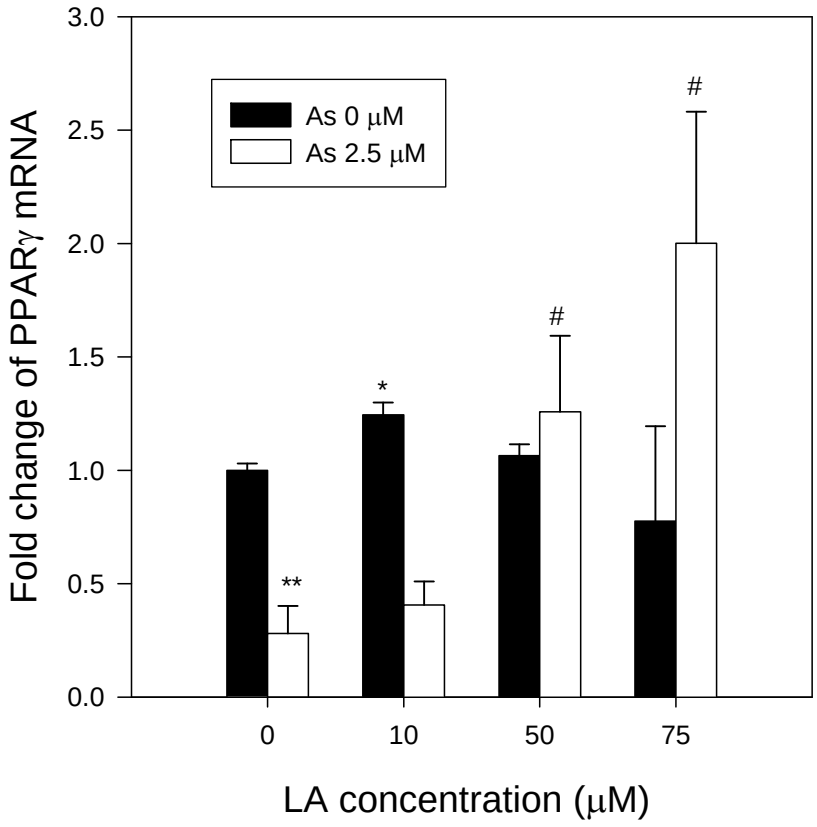
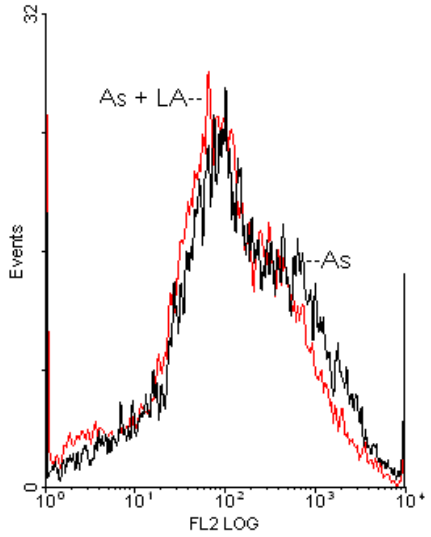


Figure 4. Effects of As and LA on PPARγ mRNA expression in PMA-differentiated THP-1 cells. Differentiation into macrophages was achieved by treating the THP-1 cells in 6-well plates ($1x10^5$ cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 nM) for 72 h. Differentiated THP-1 cells were incubated at 37 ^oC with sodium arsenite ($NaAsO_2$) (0 and 2.5 μM) alone or in combination with LA (10, 50 and 75 μM) for 24h. PPARγ mRNA expression was analyzed using RT-Q-PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. #, $p<0.05$ represents significant differences *vs.* cells treated with the same concentration of As alone. *, $p<0.05$; **, $p<0.01$ represent significant differences *vs.* normal control.

A



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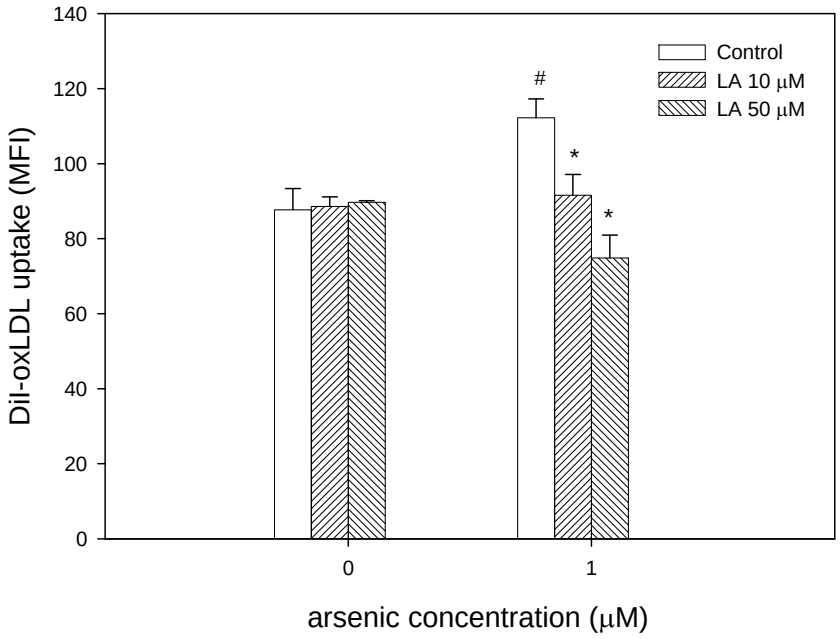

Figure 2. Effects of arsenic and α-lipolic acid (LA) on DiI-oxLDL uptake in THP-1 macrophages. Differentiated THP-1 cells were incubated at 37 ^{o}C with sodium arsenite ($NaAsO_2$) (0, 1 μM) alone or in combination with LA (10, 50 μM) for 24h. DiI-oxLDL uptake was analyzed using flow cytometry as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. #, $p<0.05$ represents significant differences *vs.* normal control. *, $p<0.05$; **, $p<0.01$ represent significant differences *vs.* cells treated with the same concentration of As alone.

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