PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2010, 37(2): 190~199
www.pibb.ac.cn

Paraoxon Down Regulates ATP-binding Cassette Transporter A1 Expression and Decreases Cholesterol Efflux Through Cyclic AMP Signaling Pathway in RAW 264.7 Macrophage-derived Foam Cells*

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Abstract ATP-binding Cassette Transporter A1 (ABCA1) plays a critical role in the reverse cholesterol transport (RCT). Previous studies showed that paraoxon, the active metabolite of organophosphorus insecticide, increased cholesterol retention in macrophages. However, its underlying mechanisms remain to be elucidated. The effect of paraoxon on ABCA1 expression and ABCA1-dependent cholesterol efflux was investigated, and then the role of cyclic adenosine monophospate (cAMP) signaling pathway in the regulation of ABCA1 expression and ABCA1-mediated cholesterol efflux was examined by paraoxon in RAW 264.7 macrophage-derived foam cells. Results showed that paraoxon significantly down regulated ABCA1 expression and reduced ABCA1-dependent cholesterol efflux and increased the levels of the total, free and esterified cholesterols in a time- and dose-dependent manner. Paraoxon also markedly reduced cAMP level and decreased adenylate cyclase (AC) activity and increased cAMP-specific phosphodiesterase (PDE) activity. Furthermore, cAMP analogs dibutyryl cyclic adenosine monophosphate (dBcAMP) markedly compensated the down-regulation of ABCA1 expression and partly compensated the reduction of ABCA1-mediated cholesterol efflux induced by paraoxon. Also, both adenylate cyclase agonist forskolin and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) markedly compensated the suppression effect on cAMP level induced by paraoxon. In conclusion, the results mentioned above suggest that paraoxon down regulates ABCA1 expression and decreases ABCA1-mediated cholesterol efflux through cyclic AMP signaling pathway in RAW 264.7 macrophage-derived foam cells.

Key words paraoxon, ABCA1, cAMP, atherosclerosis, reverse cholesterol transport
DOI: 10.3724/SP.J.1206.2009.00476

Atherosclerosis is the underlying pathologic process responsible for most cardiovascular disease. The accumulation of lipoprotein cholesterol in the artery wall is thought to be an important factor in the development of atherosclerosis[1]. Intimal macrophages take up free cholesterol from the extracellular matrix and cholesteryl esters from modified and unmodified low-density lipoprotein (LDL) particles *via* scavenger receptor type A and LDL receptors, respectively [2]. Macrophages that accumulate excessive amounts of cholesteryl esters are transformed into foam cells, which contribute to the progression of atherosclerosis. The transport of cholesterol from vessel wall macrophages to the liver is termed macrophage reverse cholesterol transport (RCT) and is proposed to be the process by which regression of atherosclerotic lesions can occur[3].

*This work was supported by grants from The National Natural Science Foundation of China (30570754), The Fund of the Department of Education of Hunan Province (08C746) and The Fund for Bring New Ideal of Central South University (ZRE52).
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Received: August 10, 2009 Accepted: December 8, 2009

Transferring cellular cholesterol to lipid-free or lipid-poor A-I apolipoprotein A-I (apoA-I) by ATP-binding cassette transporter A1 (ABCA1) is the rate-limiting step in generating plasma HDL[4]. ABCA1 is a member of a large family of ABC transporters and contains two ATP binding domains and two six helix transmembrane domains. ABCA1 expression is highly regulated at the transcriptional and post-transcriptional levels in macrophages[5]. Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger which regulates ABCA1 expression by acting both at the transcriptional and post-transcriptional level[6–7]. Cyclic AMP/protein kinase A (PKA)-dependent pathway plays an important role in ABCA1 expression and phosphorylation and cellular lipid efflux[8].

Paraoxon(O,O-diethyl-O-pnitrophenyl phosphate) is the bioactivated metabolites of organophosphorous ester (OP) pesticides and is formed mainly in liver following cytochrome P450 monooxygenase-catalyzed desulfuration[9]. Previous studies reported that paraoxon enhanced retention of intracellular cholesteryl esters in human THP-1 monocyte/macrophages [10]. However, whether paraoxon influences ABCA1 expression and ABCA1-dependent cholesterol efflux is unclear, and whether cAMP signaling pathway involves in this process has not been explored. In the present study, we investigated the effect of paraoxon on ABCA1 expression and ABCA1-mediated cholesterol efflux and examined the role of cAMP signaling pathway in the regulation of ABCA1 expression and ABCA1-mediated cholesterol efflux by paraoxon in RAW 264.7 macrophage-derived foam cells. We demonstrated that paraoxon significantly decreased ABCA1 expression and impaired ABCA1-mediated cholesterol efflux in RAW 264.7 macrophage-derived foam cells which was associated with reducing adenyl cyclase activity and increasing phosphodiesterase activity induced by paraoxon which resulted in the reduction of intracellular cAMP level.

1 Materials and methods

1.1 Materials

RPMI 1640 and TRIzol Reagent were parchased from Invitrogen (1 600 Faraday Ave, Carlsbad, USA). Hydroxyethyl piperazine ethanesulfonic acid (HEPES), fetal calf serum (FCS), forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cyclic adenosine monophosphate (dBcAMP) and paraoxon were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, 830 Harrington Court, Burlington, Ontario, Canada), DyNAmoTM SYBR® Green qPCR Kits (Finnzymes, keilaranta 16, 02150 Espoo, Finland), cAMP enzyme immunoassay (EIA) kit (Design Inc., Ann Arbor, MI, USA), rabbit anti-ABCA1 and β-actin specific antibodies (Santa Cruz Biotechnology, CA, USA). Paraoxon (1, 10, 100 μmol/L) was dissolved in ethanol.

1.2 Cell culture and foam cell formation evaluated by Oil Red O staining

Murine RAW 264.7 cells were seeded into six-well plates at 1.0×10^6 cells per well in RPMI 1640 medium containing 10% FCS, and maintained at 37℃ in a humidified atmosphere of 5% CO_2. Macrophages were transformed into foam cells by incubation with the presence of 50 mg/L oxidized LDL (ox-LDL) in serum-free RPMI 1640 medium containing 0.3% bovine serum albumin (BSA) for 48 h. Then, macrophages were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde-PBS for 10 min. After washing with 60% isopropanol, macrophages were stained with 0.3% Oil Red O in 60% isopropanol for 15 min and then washed with 60% isopropanol again. Subsequently macrophages were counterstained with hematoxylin for 4 min. After abundantly washing with water, macrophages were photographed with a microscope at 400× magnification.

1.3 Cellular cholesterol efflux experiments

Foam cells were labeled with 7.4×10^6 Bq/L [^{3}H] cholesterol and cholesterol-loaded using 50 mg/L ox-LDL. After 48 h, cells were washed with PBS for three times and incubated overnight in RPMI 1640 medium containing 0.1% (*w*/*v*) BSA to allow equilibration of [^{3}H] cholesterol in all cellular pools. Equilibrated [^{3}H] cholesterol-labeled cells were washed with PBS and incubated in 2 ml of efflux medium containing RPMI 1640 medium and 0.1% BSA with 25 mg/L human plasma apoA-I as the acceptor for cholesterol for 24 h. A 150 μl sample of efflux medium was obtained at the times designated and passed through a 0.45 μm filter to remove any floating cells. The effect of paraoxon on cellular cholesterol efflux was examined. Cells were washed twice with PBS, and cellular lipid was extracted with isopropanol. Medium and cell-associated [^{3}H] cholesterol were then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: [total media counts/(total cellular

counts + total media counts)] × 100[11].

1.4 Phospholipid efflux experiments

Foam cells were incubated with 7.4×10^6 Bq/L of [^{3}H] choline chloride to label the phospholipids. After 48 h, cells were then washed with PBS for three times and incubated overnight in RPMI 1640 medium containing 0.1% (*w*/*v*) BSA. After 8 h of incubation with medium with 10 g/L apoA-I, efflux medium was collected, centrifuged to remove cell debris, and aliquots were taken for extraction and separated by thinlayer chromatography with the use of silica G plates developed in chloroform/methanol/ammonia (25%, *w*/*v*) / water (50∶65∶5∶4, *v*/*v*/*v*/*v*). Phospholipids spots were visualized by I_2 vapors and identified by comigration with standards. Relative radioactivity was determined by Phosphoscreen and quantified by PhosphorImager. Phospholipid efflux was expressed as percent counts in the supernatant versus total for each individual lipid[8].

1.5 cAMP level assays

RAW 264.7 macrophage-derived foam cells (4×10^5) were plated in a 24-well plate. After treatment with paraoxon, incubation media was aspirated and placed in microcentrifuge tubes. The cells were lysed by the addition of 500 μl of 0.1 mol/L HCl containing 0.2% Triton X-100 and incubated for 10 min at room temperature. The lysed cells were scraped into microcentrifuge tubes. The samples were centrifuged at 1 300 *g* for 5 min at room temperature. cAMP concentrations in these supernatants were measured with an EIA assay kit in accordance with the manufacturer's instructions. cAMP concentrations in the culture media were also measured. The total concentration of cAMP produced by each condition was obtained by adding cellular and culture media cAMP contents.

1.6 Adenylate cyclase (AC) and cAMP-specific phosphodiesterase (PDE) assay

Plasma membrane preparations were isolated by differential centrifugation from RAW 264.7 macrophage derived foam cells. Cells were homogenized with a homogenizer in 25 mmol/L HEPES, 0.25 mol/L sucrose, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 5 mmol/L benzamidine, and a protease inhibitor cocktail (1∶400). Membranes were collected by two steps of differential centrifugation (1 000 *g* for 5 min at 4℃ and 40 000 *g* for 25 min at 4℃), and the protein concentrations in the samples were examined using BCA kit. For AC assay, 30 μg of membrane protein was added to reaction buffer [55 mmol/L Tris-HCl (pH 7.4), 5.0 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L ATP, 0.1 μmol/L GTP, 0.2 U pyruvate kinase, 0.1 U myokinase, and 2.5 mmol/L phosphoenolpyruvate] and incubated for 15 min at 37℃. The converted cAMP from ATP in the supernatant of the samples was determined with an EIA assay kit[8, 12]. cAMP-specific phosphodiesterase assay was performed according to the following method. RAW macrophage-derived foam cells were scraped into lysis buffer [25 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 1∶400 protease inhibitor cocktail], homogenized, and then sonicated for 30 s on ice. Cells lysates were centrifuged (14 000 *g* for 5 min at 4℃), and 30 μg protein of cell lysates was incubated in reaction buffer[25 mmol/L HEPES(pH 7.4), 90 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.75 mmol/L $CaCl_2$, and 100 nmol/L cAMP]. The reaction was terminated by the addition of 0.1 mol/L HCl and centrifuged. The cAMP remaining in the supernatants was measured by an EIA kit. The PDE activity was determined as the amount of cAMP hydrolyzed during the reaction[13].

1.7 High performance liquid chromatography assays

Cultural cells were washed with PBS for three times. The proper volume (about 1 ml) of 0.5% NaCl was added to 50～200 μg cellular proteins per ml. Cells were sonicated using an ultrasonic processor for 3 min. The protein concentration in cell lysate solution was measured using BCA kit. 0.1 ml aliquot cell solution (containing 5～20 μg protein) was used to measure the free cholesterol (FC), and another aliquot for total cholesterol (TC) measurement. FC was dissolved in isopropanol (1 g/L) and stored at –20℃ as stock solution. Cholesterol standard calibration solution ranging from 0 to 40 μg of cholesterol per ml was obtained by diluting the cholesterol stock solution. 0.1 ml of each sample (cholesterol standard calibration solutions, or cell lysate solutions) was supplemented with 10 μl reaction mixture including 500 mmol/L $MgC1_2$, 500 mmol/L Tris-HCl (pH 7.4), 10 mmol/L dithiothreitol, and 5% NaCl. 0.4 U cholesterol oxidase in 10 μl 0.5% NaCl was added to each tube for free cholesterol determination, or 0.4 U cholesterol oxidase plus 0.4 U of cholesterol esterase for TC measurement. The total reaction solution in tube was incubated at 37℃ for 30 min, and 100 μl methanol∶ethanol (1∶1) was added to stop the reaction. Each solution was kept cold for 30 min to allow protein precipitation, and

centrifuged at 1 500 r/min for 15 min at 15℃. 10 μl of supernatant was applied onto a System Chromatographer (PerkinElmer Inc.) including a PerkinElmer series 200 vacuum degasser, a pump, a PerkinElmer series 600, and a PerkinElmer series 200 UV/vis detector and a disovery C-18 column. The column was eluted using isopropanol∶n-heptane∶acetonitrile (35∶13∶52) at a flow rate of 1 ml/min for 10 min. Absorbance at 216 nm was monitored. Data were analyzed with TotalChrom software from PerkinElmer[14-15].

1.8 RNA isolation and real-time quantitative PCR analysis

Total RNA from cells was extracted by using TRIzol reagent according to the manufacturer's instructions. Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on Roche Light Cycler Run 5.32 Real-Time PCR System. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the $\Delta\Delta C_t$ method and expression of β-actin was used as the internal control.

1.9 Western blot analyses

Cells were harvested and protein extracts were prepared. They were then subjected to Western blot analysis [10% SDS-polyacrylamide (SDS-PAGE); 30 μg protein per lane] using rabbit anti-ABCA1. Immunoreactivity was detected by ECL test. Protein content was calculated by densitometry using Labwords analysis software.

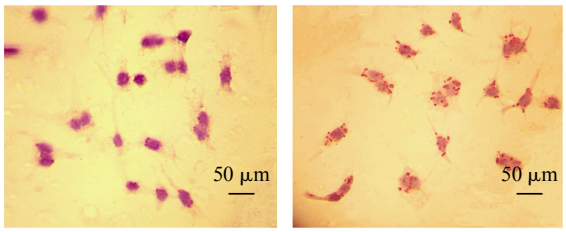
1.10 Statistical analysis

Data are expressed as $\bar{x} \pm s$. Results were analyzed by one-way ANOVA and Student's t test, using SPSS 14.0 software. $P < 0.05$ was regarded as significant.

2 Results

2.1 Formation of RAW 264.7 macrophage-derived foam cells

RAW 264.7 macrophages were incubated with 50 mg/L ox-LDL for 48 h to induce foaming. Cells were then stained with Oil Red O and observed using a microscope. Lipid droplets appeared in cytoplasm (Figure 1). The results of high performance liquid chromatography (HPLC) analysis showed that the TC, FC and cholesterol ester (CE) in RAW 264.7 microphage-derived foam cells were significantly increased compared with that of the RAW 264.7 macrophages (Table 1). Cholesterol ester in foam cells increased to about 629% of that of the RAW 264.7 macrophages (321 mg/g protein *vs*. 51 mg/g protein). The results suggested that macrophages had accumulated considerable lipids in their cytoplasm.



RAW 264.7 macrophages　RAW 264.7 macrophage-derived cells

Fig. 1　Oil red O dyeing in RAW 264.7 macrophages and RAW 264.7 macrophage-derived foam cells

RAW 264.7 macrophages were divided into two groups and cultured in medium at 37℃ containing 50 mg/L ox-LDL for 48 h. The red color indicated the lipid droplets (both free cholesterol and cholesterol ester) stained by the Oil red O. The blue color indicated the nucleus stained by hematoxylin. RAW 264.7 macrophate-derived foam cells were full of the cholesterol.

Table 1　The levels of the total, free and esterified cholesterols in RAW 264.7 macrophages and RAW 264.7 macrophage-derived foam cells

Groups	TC	FC	CE	CE/TC
RAW 264.7 cells	146±12	95±7	51±5	34.9%
Foam cells	513±37*	192±20*	321±28*	62.6%*

RAW 264.7 macrophages were divided into two groups and cultured in medium at 37℃ containing 50 mg/L ox-LDL for 48 h. Cellular cholesterol and cholesterol ester were extracted. HPLC was performed to examine the cellular total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE). Cholesteryl ester, total and free cholesterol levels are expressed as mg/mg protein. Data are expressed as $\bar{x} \pm s$. from three independent experiments, each performed in triplicate. *$P < 0.05$ *vs*. RAW 264.7 cells group.

2.2 Effect of paraoxon on cholesterol level and efflux in RAW 264.7 macrophage-derived foam cells

RAW 264.7 macrophage-derived foam cells derived from oxidized LDL were treated with 0, 1, 10, 100 μmol/L paraoxon for 24 h and were treated with 5 g/L BSA for 24 h or with 100 μmol/L for 0 h, 6 h, 12 h, 24 h respectively. Foam cells were cultured in efflux medium containing RPMI 1640 medium and 0.1% BSA with 25 mg/L human plasma apoA-I as the acceptor for cholesterol, then cells were stained with Oil red O. The lipid droplet contents in cells treated with 10 and 100 μmol/L paraoxon for 24 h and treated with 100 μmol/L paraoxon for 12 h and 24 h were

increased obviously compared with the control group (data not shown). As shown in Table 2, the TC, FC and CE in RAW 264.7 macrophage-derived foam cells were significantly increased after treated with 10 and 100 μmol/L paraoxon for 24 h compared with that of the control group. Likewise, the TC, FC and CE in RAW 264.7 macrophage-derived foam cells were significantly increased by treatment with 100 μmol/L paraoxon for 12 h or 24 h compared with that of 0 h group (Table 3).

Table 2 Effect of paraoxon on the total, free and esterified cholesterols in RAW 264.7 macrophage-derived foam cells

Groups	TC	FC	CE	CE/TC
Control	507±37	189±20	318±28	62.7%
1 μmol/L paraoxon	523±36	197±19	326±31	62.3%
10 μmol/L paraoxon	675±48*	264±22*	411±34*	60.9%
100 μmol/L paraoxon	761±61*	298±35*	463±44*	60.8%

RAW 264.7 macrophage-derived foam cells were divided into four groups and cultured in medium at 37℃ containing 0, 1, 10 and 100 μmol/L paraoxon for 24 h respectively. Cellular cholesterol and cholesterol ester (CE) were extracted. HPLC was performed to determine the cellular total cholesterol (TC), free cholesterol (FC) and free cholesterol (CE). Control refers to vehicle-treated foam cells. TC, FC and CE levels are expressed as mg/mg protein. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. * $P < 0.05$ *vs*. control group.

Table 3 Effect of paraoxon on the total, free and esterified cholesterols in RAW 264.7 macrophage-derived foam cells

Groups	TC	FC	CE	CE/TC
BSA(24 h)	522±35	197±21	325±29	62.3%
0 h	518±36	191±20	327±31	62.3%
6 h	567±41	225±21	342±29	60.3%
12 h	669±54*	226±31*	413±46*	61.7%
24 h	758±53*	291±33*	467±48*	61.6%

RAW 264.7 macrophage-derived foam cells were divided into four groups and cultured in medium at 37℃ containing 5 g/L bovine serum albumin (BSA) for 24 h and cultured in medium containing 100 μmol/L paraoxon for 0 h, 6 h, 12 h, 24 h respectively. Cellular cholesterol and cholesterol ester (CE) were extracted as described above. And HPLC was performed to determine the cellular total cholesterol (TC), free cholesterol (FC) and CE. TC, FC and CE levels are expressed as mg/mg protein. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. * $P < 0.05$, *vs*. 0 h group.

Cholesterol efflux was determined as described in "**Materials and methods**". The cholesterol efflux mediated by apoA-I of RAW 264.7 macrophages-derived foam cells was significantly decreased by treatment with 10 or 100 μmol/L paraoxon for 24 h (Figure 2a) and 100 μmol/L paraoxon for 12 h or 24 h (Figure 2b). RAW 264.7 macrophage-derived foam cells were incubated with [^{3}H] choline chloride to label the phospholipids. Paraoxon also resulted in significant reduction of apoA-I-mediated phospholipids efflux (Figure 2c and d).

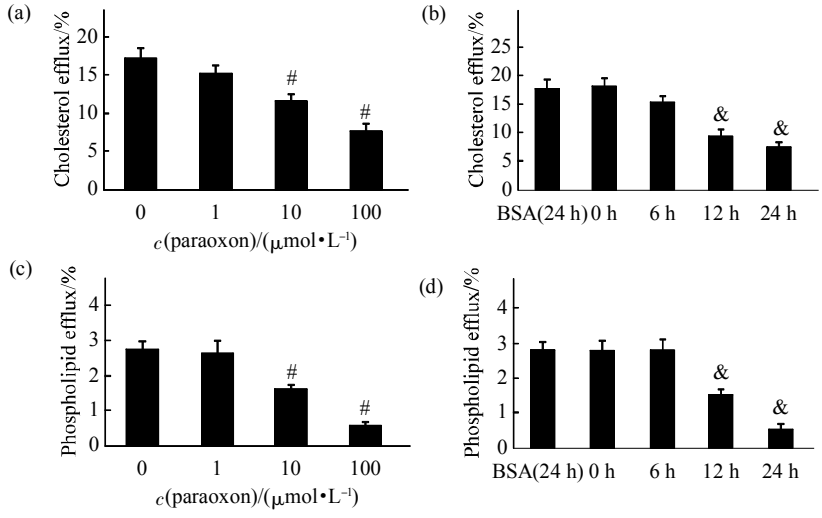


Fig. 2 Effect of paraoxon on cholesterol and phospholipids efflux in RAW 264.7 macrophage-derived foam cells

Foam cells were treated with 0, 1, 10, 100 μmol/L paraoxon for 24 h respectively (a and c), and foam cells were treated with 5 g/L BSA for 24 h or with 100 μmol/L paraoxon for 0 h, 6 h, 12 h, 24 h (b and d) respectively. Cellular cholesterol and phospholipids efflux were analyzed as shown above. Control refers to vehicle-treated foam cells. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. $^{\#}P < 0.05$, *vs*. control group. $^{\&}P < 0.05$, *vs*. 0 h group.

2.3 ABCA1 expression was down-regulated by paraoxon in RAW 264.7 macrophage-derived foam cells

ABCA1 is a key player in reverse cholesterol transport and cellular cholesterol homeostasis. Cholesterol efflux from RAW macrophage-derived foam cells was decreased by paraoxon, so we next investigated the effect of paraoxon on ABCA1 expression in RAW 264.7 macrophage-derived foam cells by real-time quantitative PCR and Western immunoblotting assays. Paraoxon significantly decreased ABCA1 mRNA (Figure 3a and b) and protein (Figure 3c and d) expression in a dose-dependent and time-dependent manner.

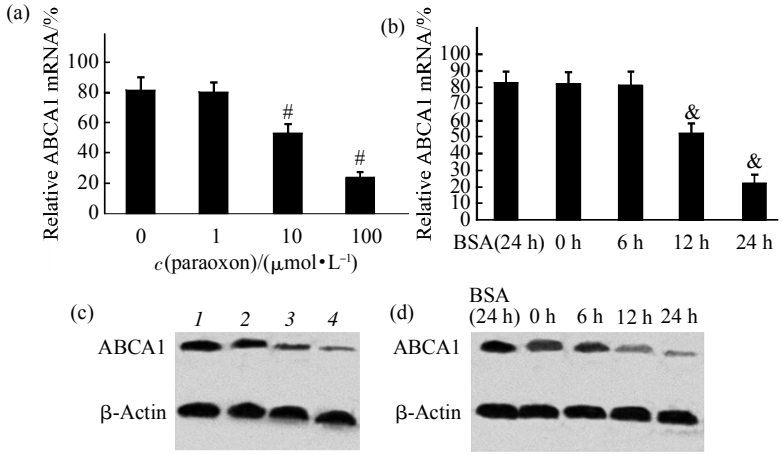


Fig. 3 Effect of paraoxon on expression of ABCA1 in RAW 264.7 macrophage-derived foam cells

Foam cells were treated with 0, 1, 10, 100 μmol/L paraoxon for 24 h respectively (a and c), and foam cells were treated with 5 g/L BSA for 24 h or with 100 μmol/L paraoxon for 0 h, 6 h, 12 h, 24 h (b and d) respectively. ABCA1 mRNA expression was measured by real-time quantitative PCR (a and b). ABCA1 protein expression was measured by Western blot analyses (c and d). Control refers to vehicle-treated foam cells. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. $^{\#}P < 0.05$, *vs*. control group. $^{\&}P < 0.05$, *vs*. 0 h group. *1*: Control; *2*: 1 μmol/L; *3*: 10 μmol/L; *4*: 100 μmol/L.

2.4 cAMP is involved in down-regulation of ABCA1 expression and decrease of cholesterol efflux induced by paraoxon in RAW 264.7 macrophage-derived foam cells

ABCA1 is the cAMP-inducible apolipoprotein receptor that promotes secretion of lipids from macrophages [16]. To confirm whether the cAMP is involved in the regulation of ABCA1 expression and ABCA1-dependent cholesterol efflux by paraoxon, we investigated effect of paraoxon on the cAMP level. The results showed that the level of cAMP was markedly attenuated when cells were treated with paraoxon (Figure 4a and b). We then investigated the effect of cAMP analogs dBcAMP on the down-regulation of ABCA1 expression and cholesterol efflux by paraoxon. As shown in Figure 4c, the down-regulation of ABCA1 expression by paraoxon was significantly compensated by treatment with dBcAMP. While reduction of cholesterol efflux induced by paraoxon was partly abolished by addition of dBcAMP (Figure 4d).

2.5 Adenylate cyclase and cAMP-specific phosphodiesterase are involved in down-regulation of ABCA1 expression and decrease of cholesterol efflux induced by paraoxon

Intracellular cAMP metabolism is determined primarily by activities of adenylate cyclase (AC) and phosphodiesterase (PDE)[17]. To test whether paraoxon effects production and hydrolysis of cAMP, the effect of paraoxon on adenylate cyclase and cAMP-specific phosphodiesterase activities in RAW 264.7 macrophage-derived foam cells was examined. As shown in Figure 5a, b, c and d, AC activity was decreased obviously by paraoxon and cAMP-specific PDE activity was increased significantly by paraoxon in a dose- and time-dependent manner. We then investigated effect of AC agonist forskolin and cAMP-specific PDE inhibitor IBMX on the down-regulation of cAMP level by paraoxon. As shown in Figure 5e, the inhibitory effect of paraoxon on cAMP level was markedly compensated by treatment with forskolin, while treatment of cells with IBMX notablly abolished the suppression effect of paraoxon (Figure 5f).

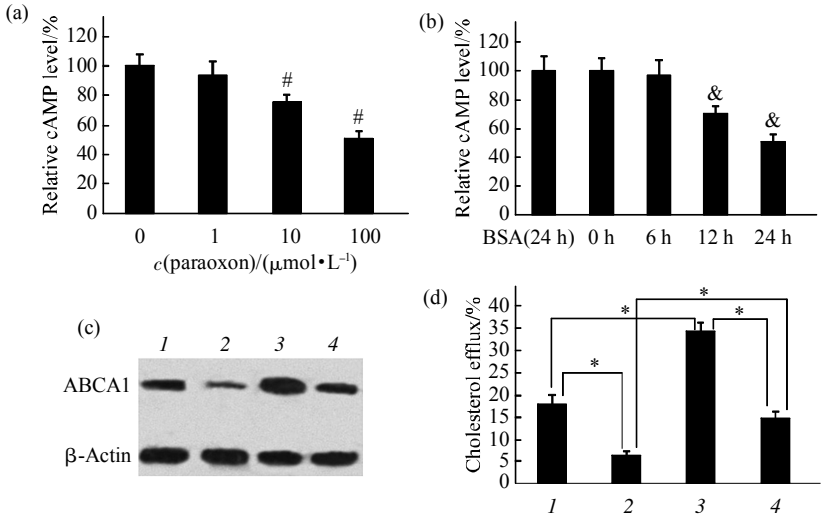


Fig. 4　The cAMP level was decreased by paraoxon in RAW 264.7 macrophage-derived foam cells

Foam cells were treated with 0, 1, 10, 100 μmol/L paraoxon for 24 h (a) and 5 g/L BSA for 24 h or with 100 μmol/L paraoxon for 0 h, 6 h, 12 h, 24 h respectively (b). (c) Effect of dBcAMP on protein expression of ABCA1 in RAW macrophage-derived foam cells. *1*: Control; *2*: Paraoxon; *3*: dBcAMP; *4*: Paraoxon + dBcAMP. (d) Foam cells were treated with 100 μmol/L paraoxon, 1.0 mmol/L dBcAMP, and 100 μmol/L paraoxon + 1.0 mmol/L dBcAMP for 24 h respectively. *1*: Control; *2*: Paraoxon; *3*: dBcAMP; *4*: Paraoxon + dBcAMP. Control refers to vehicle-treated foam cells. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. $^{\#}P < 0.05$, *vs*. control group. $^{\&}P < 0.05$, *vs*. 0 h group. $^{*}P < 0.05$.

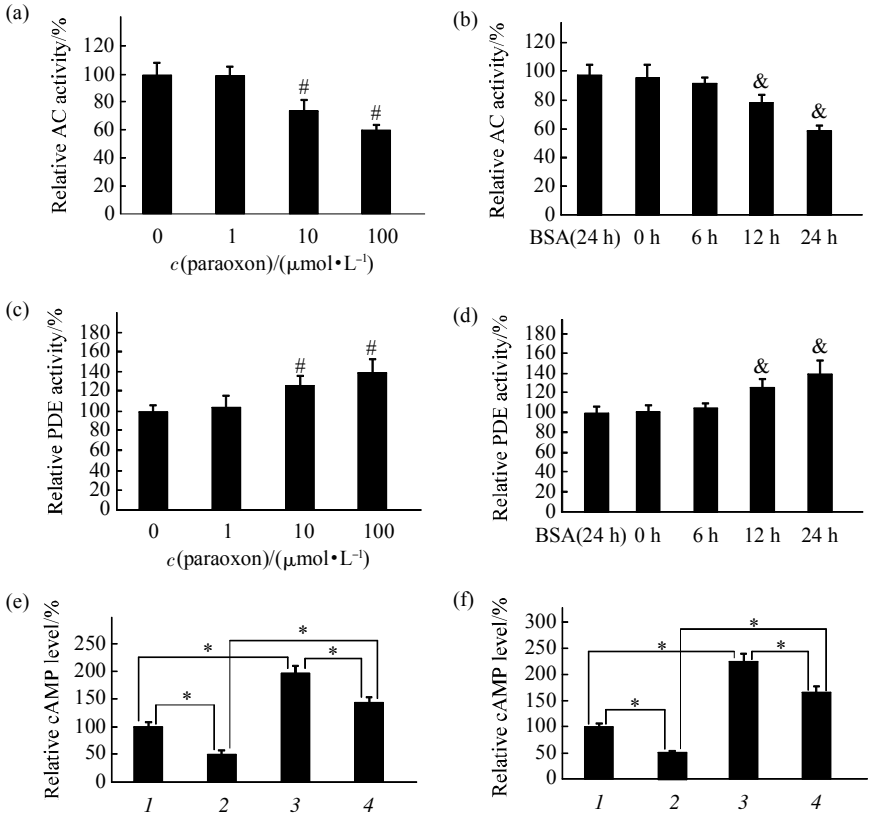


Fig. 5　AC and cAMP-specific PDE are involved in cAMP decreased by paraoxon

(a, c) Foam cells were treated with 0, 1, 10, 100 μmol/L paraoxon for 24 h. (b, d) Foam cells were treated with 5 g/L BSA for 24 h or with 100 μmol/L paraoxon for 0 h, 6 h, 12 h, 24 h respectively. (e) Foam cells were treated with 100 μmol/L paraoxon, 40 μmol/L forskolin, and 100 μmol/L paraoxon + 40 μmol/L forskolin for 30 min at 37℃ respectively. *1*: Control; *2*: Paraoxon; *3*: Forskolin; *4*: Paraoxon + Forskolin. (f) Foam cells were treated with 100 μmol/L paraoxon, 0.2 μmol/L IBMX, and 100 μmol/L paraoxon+ 0.2 μmol/L IBMX for 30 min at 37℃ respectively. *1*: Control; *2*: Paraoxon; *3*: IBMX; *4*: Paraoxon + IBMX. Control refers to vehicle-treated foam cells. Data are expressed as $\bar{x} \pm s$ from three independent experiments, each performed in triplicate. $^{\#}P < 0.05$, *vs*. control group. $^{\&}P < 0.05$, *vs*. 0 h group. $^{*}P < 0.05$.

3 Discussion

Macrophages contribute significantly to the development and progression of atherosclerosis. Macrophages in arterial walls scavenge free cholesterol and cholesteryl esters in LDL particles and become engorged with lipids. The excessive accumulation of cholesterol in arterial wall macrophages can result in foam cell formation and the progression of atherosclerosis. Macrophages are also believed to play an important role in the regression of atherosclerosis by the process of macrophage reverse cholesterol transport[18]. ABCA1 plays a critical role in the process of in reverse cholesterol transport and promotes the transport of cholesterol and phospholipids to apoA-I, which plays a central role in the process of atherosclerosis[19]. In the present study, we demonstrated that paraoxon significantly down- regulated ABCA1 expression and decreased ABCA1-mediated cholesterol efflux through cAMP signaling pathway in RAW 264.7 macrophage-derived foam cells.

Paraoxon is the active metabolites of the most commonly used or well-studied OP pesticides and is formed mainly in liver following cytochrome P450 monooxygenase-catalyzed desulfuration. In accordance with previous studies [10], our results showed that paraoxon reduced cholesterol efflux and increased the total cholesterol, free cholesterol and cholesterol ester in RAW 264.7 macrophage-derived foam cells.

ABCA1 is a key player in reverse cholesterol transport and in regulating cellular cholesterol homeostasis. A growing body of evidence indicates that ABCA1 not only plays a major role in HDL biogenesis and RCT process, but also has emerged as potential targets for therapies designed to inhibit the development of atherosclerotic vascular disease [20–21]. ABCA1 is regulated both at the transcriptional level *via* liver and retinoid X receptors and at the post-transcriptional level via changes in trafficking and the turnover rate of ABCA1 protein[21–22]. In the current study, we also showed that paraoxon obviously down-regulated ABCA1 mRNA and protein expression and decreased ABCA1-mediated cholesterol efflux in a dose-dependent and time-dependent manner. cAMP is a ubiquitous second messenger, which up regulates ABCA1 expression by acting both at the transcriptional and post-transcriptional level[23–24]. Olivier *et al*.[25] reported that paraoxon inhibited cAMP formation through muscarinic receptor-dependent and independent mechanisms. In our study, we demonstrated that the level of cAMP was markedly decreased when cells were treated with paraoxon, and also found that cAMP analogs dBcAMP significantly compensated the down-regulation of ABCA1 expression induced by paraoxon, but partly compensated the reduction of cholesterol efflux induced by paraoxon. These results suggest that paraoxon decrease cholesterol efflux and increase cholesterol retention in macrophages through several potential manner. For example, Crow *et al*.[10] reported that paraoxon reduced cholesteryl ester mobilization and increased cholesterol retention by inhibition of carboxylesterase 1, which resulted in the inhibition of macrophage RCT.

Intracellular level of cAMP is regulated by both the adenylyl cyclase that synthesizes cAMP from adenosine triphosphate (ATP) and the cAMP phosphodiesterases that degrade cAMP to 5′-AMP[26–27]. Organophosphorus compounds had been shown to interact directly with muscarinic receptors coupled to inhibition of adenylyl cyclase[28–29]. Our current results indicated that adenyl cyclase (AC) activity was decreased obviously and cAMP-specific phosphodiesterase (PDE) activity was increased markedly by paraoxon in a dose- and time-dependent manner. Our results also showed that paraoxon-induced suppression effect on cAMP level was significantly compensated when cells were treated with AC agonist forskolin and was significantly abolished when cells were treated with cAMP-specific PDE inhibitor IBMX. These results suggest that intracellular level of cAMP is regulated by paraoxon through reducing adenylyl cyclase activity and increasing cAMP-specific PDE activity. cAMP is important intracellular second messenger which is involved in several signaling pathways. PKA is a major downstream target protein of cAMP. cAMP is an activator of PKA, which is known to stimulate cholesterol efflux and ABCA1 expression and phosphorylation[6, 30]. Previous studies also showed that the cAMP/PKA signaling pathway was involved in the regulation of ABCA1 expression and ABCA1-dependent cholesterol efflux induced by eicosapentaenoic acid in THP-1 macrophage-derived foam cells[6,8]. Our results showed that paraoxon reduced adenylyl cyclase activity and increased cAMP-specific PDE activity, which resulted in the reduction of intracellular levels of cAMP, thus we presume that paraoxon may inhibit the action of PKA. Therefore, the down-regulation of

ABCA1 expression and the reduction of ABCA1-mediated cholesterol efflux induced by paraoxon may be mediated by the cAMP/PKA signaling pathway in RAW 264.7 macrophage-derived foam cells. But further studies are needed to examine these possibilities.

Increasingly, environmental toxicants are being recognized as etiological agents that contribute to atherosclerosis [31-32]. Our results clearly show that paraoxon is able to affect cholesterol efflux and increase retention of intracellular cholesterol and cholesteryl esters in RAW 264.7 macrophage-derived foam cells. This study suggest that exposure to OP pesticides may adversely affect macrophage cholesterol efflux and increase the risk of atherosclerosis.

In conclusion, we have provided strong evidence that paraoxon may down regulate the expression of ABCA1 and decrease ABCA1-dependent cholesterol efflux through a cAMP signaling pathway in RAW 264.7 macrophage-derived foam cells.

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对氧磷降低 RAW264.7 巨噬细胞源性泡沫细胞 ABCA1 表达和胆固醇流出 *

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摘要　三磷酸腺苷结合盒转运体 A1(ABCA1)是体内胆固醇逆向转运的关键环节．对氧磷是广泛使用的有机磷农药的活性代谢产物．研究发现，对氧磷能增加巨噬细胞中胆固醇的堆积，但具体机制还不清楚．以 RAW264.7 巨噬细胞源性泡沫细胞为研究对象，观察对氧磷对 RAW264.7 巨噬细胞源性泡沫细胞 ABCA1 表达和胆固醇流出的影响并探讨其机制．结果显示，对氧磷以时间和剂量依赖的方式增加 RAW264.7 巨噬细胞源性泡沫细胞中总胆固醇、游离胆固醇和胆固醇酯水平，降低 ABCA1 表达和胆固醇流出，同时对氧磷降低细胞中环磷酸腺苷(cAMP)的水平及腺苷酸环化酶(AC)的活性和增加磷酸二酯酶(PDE)的活性，而 cAMP 的类似物双丁酰环腺苷酸(dBcAMP)能够阻断对氧磷降低 ABCA1 表达和部分阻断对氧磷降低胆固醇流出，对氧磷导致的 cAMP 水平的降低也可被 AC 激动剂福斯高林(Forskolin)和 PDE 抑制剂 3- 异丁基 -1- 甲基黄嘌呤(IBMX)所阻断．以上结果表明，对氧磷通过 cAMP 信号通路下调 RAW264.7 巨噬细胞源性泡沫细胞 ABCA1 的表达，降低细胞内胆固醇流出和增加细胞内胆固醇堆积．

关键词　对氧磷，三磷酸腺苷结合盒转运体 A1，环磷酸腺苷，动脉粥样硬化，逆向胆固醇转运
学科分类号　R363　　**DOI:** 10.3724/SP.J.1206.2009.00476

* 国家自然科学基金(30570754), 湖南省教育厅(08C746)和中南大学创新基金(ZRE52)资助项目.
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收稿日期：2009-08-10，接受日期：2009-12-08