Original Paper

High Uric Acid Activates the ROS-AMPK Pathway, Impairs CD68 Expression and Inhibits OxLDL-Induced Foam-Cell Formation in a Human Monocytic Cell Line, THP-1

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Key Words

High uric acid • Monocytes/macrophages • ROS-AMPK pathway • CD68 • Foam cell formation

Abstract

Background/Aims: Hyperuricemia is part of the metabolic-syndrome cluster of abdominal obesity, impaired glucose tolerance, insulin resistance, dyslipidemia, and hypertension. Monocytes/macrophages are critical in the development of metabolic syndrome, including gout, obesity and atherosclerosis. However, how high uric acid (HUA) exposure affects monocyte/macrophage function remains unclear. In this study, we investigated the molecular mechanism of HUA exposure in monocytes/macrophages and its impact on oxidized low-density lipoprotein (oxLDL)-induced foam-cell formation in a human monocytic cell line, THP-1. **Methods:** We primed THP-1 cells with phorbol-12-myristate-13-acetate (PMA) for differentiation, then exposed cells to HUA and detected the production of reactive oxygen species (ROS) and analyzed the level of phospho-AMPK α . THP-1 cells were pre-incubated with Compound C, an AMPK inhibitor, or N-acetyl-L-cysteine (NAC), a ROS scavenger, or HUA before PMA, to assess CD68 expression and phospho-AMPK α level. PMA-primed THP-1 cells were pre-treated with oxLDL before Compound C and HUA treatment. Western blot analysis was used to examine the levels of phospho-AMPK α , CD68, ABCG1, ABCA1, cyclooxygenase-2

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(COX-2) and NF- κ B (p65). Flow cytometry was used to assess ROS production and CD68 expression in live cells. Oil-red O staining was used to observe oxLDL uptake in cells. **Results:** HUA treatment increased ROS production in PMA-primed THP-1 cells; NAC blocked HUA-induced oxidative stress. HUA treatment time-dependently increased phospho-AMPK α level in PMA-primed THP-1 cells. The HUA-induced oxidative stress increased phospho-AMPK α levels, which was blocked by NAC. HUA treatment impaired CD68 expression during cell differentiation by activating the AMPK pathway, which was reversed by Compound C treatment. Finally, HUA treatment inhibited oxLDL uptake in the formation of foam cells in THP-1 cells, which was blocked by Compound C treatment. HUA treatment significantly increased the expression of ABCG1 and reversed the oxLDL-reduced ABCG1 expression but did not affect the expression of ABCA1, NF- κ B (p65) or COX-2. **Conclusions:** HUA exposure activated the ROS-AMPK pathway, impaired CD68 expression, and inhibited oxLDL-induced foam-cell formation in a human monocytic cell line, THP-1.

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Introduction

Uric acid is generated during nucleotide and adenosine triphosphate metabolism and is an end-product of purine metabolism in uricase mutation in humans [1]. Moreover, as compared with other mammals, in humans, defective urease activity results in increased serum uric acid level [2], so humans with purine metabolism disorder are at risk of hyperuricemia. The upper normal range of serum uric acid concentration is 360 μ mol/l for women and 400 μ mol/l for men [3]. Several clinical epidemiological studies have shown that hyperuricemia has become a prevalent disease worldwide over the past several decades because of improved standard of living [4-7].

An early study suggested that hyperuricemia is part of the metabolic-syndrome cluster of abdominal obesity, impaired glucose tolerance, insulin resistance, dyslipidemia, and hypertension, thereby resulting in hemodynamic abnormalities [8, 9]. However, the precise mechanism is still unknown. In recent decades, increasing evidence has revealed that hyperuricemia is a major risk factor for gout but also other metabolic disorders, including metabolic syndrome, hypertension, atherosclerosis, chronic renal diseases, and peripheral vascular diseases [10-13]. A recent animal study indicated that elevated serum uric acid level exacerbates left ventricular diastolic dysfunction in mice fed a Western diet, which also leads to other pathophysiological processes, including myocardial hypertrophy, oxidative stress, interstitial fibrosis and macrophage proinflammatory polarization [14, 15]. Monocytes/macrophages have critical importance in the development of metabolic syndrome, including gout, obesity-related diseases, insulin resistance and atherosclerosis [12, 16-19]. However, the relationship between high uric acid (HUA) level and monocyte/macrophage activity remains unclear.

The glycoprotein CD68 binds to low-density lipoprotein (LDL) and is mainly expressed in cytoplasm [20, 21]. Some studies have shown that CD68 is a marker of activated macrophages and plays a key role in oxidized-LDL (oxLDL) uptake, which is related to foam-cell formation [22, 23]. In addition, AMP-activated protein kinase (AMPK), as a "metabolic master switch" [24-26], is involved in promoting anti-inflammatory polarization in lipopolysaccharide (LPS)-challenged macrophages [27, 28]. Whether AMPK has a role in the effect of HUA level on CD68 expression is unknown.

HUA level can induce oxidative stress in multiple cells [29-33]. Our previous study indicated that HUA level induces oxidative stress in both hepatocytes and pancreatic β cells, which results in insulin resistance and growth inhibition, respectively [34, 35]. We hypothesized that HUA level can increase reactive oxygen species (ROS) production in human monocytes/macrophages. Here, we investigated the effect of HUA treatment on ROS production, AMPK activity and CD68 expression during the differentiation of phorbol-12-myristate-13-acetate (PMA)-primed human monocytic THP-1 cells. The impact of HUA

exposure on oxLDL-induced foam-cell formation in THP-1cells and the mechanism were also studied.

Materials and Methods

Reagents

Anti-phospho-AMPK α (Thr-172) and anti-AMPK antibodies were from CST (St. Louis Park, MN, USA). Anti-CD68 and anti-NF- κ B p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-GAPDH, anti-ABCA1, anti-ABCG1, and anti-cyclooxygenase-2 (anti-COX-2) antibodies were from Abcam (UK). Rabbit anti- β -actin antibody was from Bioworld (St. Louis Park, MN, USA). Uric acid, Compound C, hydrofluorescein diacetate (DCFH-DA), and Oil-red O were from Sigma (St. Louis, MO, USA). Anti-human CD68 FITC was from eBioscience (San Diego, CA, USA). N-acetyl-L-cysteine (NAC) was from ENZO Life Sciences (Farmingdale, NY, USA). PMA was from Beyotime (Shanghai). OxLDL was from Yiyuan Biotech (Guangzhou). Fixation buffer and permeabilization wash buffer were from 4A Biotech (Beijing). All other chemicals and solvents were of analytical grade. For primary buffer, uric acid stock solution was prepared at 15 mg/ml in 0.5 M NaOH. NAC was prepared at 500 mM in ultrapure water.

Cell culture and treatment

THP-1 cells were obtained from the American Type Cell Collection, grown in RPMI-1640 Medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin (complete medium) and maintained at 37°C in a humidified, 5% CO₂ environment with medium changed every 2 days. To assess AMPK (Thr-172) phosphorylation, cells (5×10^5) were primed with 160 nM PMA for 24 h, then exposed to uric acid (15 mg/dl) for different times (0, 30, 60, 120 min), harvested by scraping, and stored at -80°C. In experiments with an inhibitor, cells were pre-treated with the antioxidant NAC (10 or 20 mM) for 1 h before co-incubation with PMA for 24 h, then exposed to uric acid (15 mg/dl) for 2 h. To measure intracellular ROS levels, cells were sub-cultured in 6-well plates (2.5×10^5 cells/well) for treatment as follows: control, vehicle (NaOH), NAC alone (10 mM), HUA alone (uric acid, 15 mg/dl $\times$ 2 h), HUA+10 NAC (10 mM $\times$ 24 h), HUA+20 NAC (20 mM $\times$ 24 h), then stained with 10 μ M DCFH-DA for 30 min at 37°C. Stained cells were viewed by fluorescence microscopy and analyzed by use of the BD Accuri™ C6 Flow Cytometer. To detect CD68 expression, cells were pre-incubated with Compound C (10 μ M) for 1 h, then exposed to uric acid (15 mg/dl) for 2 h before co-incubation with PMA (160 nM) for 24 h. To assess the expression of other proteins (ABCG1 and ABCA1) relative to lipid metabolism, PMA-primed cells were pre-incubated with uric acid (15 mg/dl) for 30 min, then co-incubated with oxLDL (50 mg/L) for 24 h.

Flow cytometry of CD68 expression

THP-1 cells were sub-cultured in 6-well plates (2.5×10^5 cells/well) and pretreated with Compound C (10 μ M) as described previously. Cells were then collected into tubes, fixed with fixation buffer and permeabilized with permeabilization wash buffer, then stained with anti-human CD68 FITC at 4°C for 30 min. Stained cells were analyzed by use of the BD Accuri™ C6 Flow Cytometer.

Western blot analysis

Cells (5×10^5) were sonicated in 300 μ l RIPA buffer supplemented with protease and phosphatase inhibitor (3 μ l), NaF (1 mM, 3 μ l), sodium orthovanadate (1 mM, 3 μ l), and PMSF (1 mM, 3 μ l), homogenized, then underwent centrifugation (12,000 g for 5 min). The supernatant was used for protein determination by use of the BCA Protein Assay Kit (Pierce, IL, USA). Equal amounts of total protein underwent 8-10% SDS-PAGE, then were transferred to polyvinylidene difluoride membranes (Millipore, Shanghai), which were blocked with 5% non-fat milk and incubated with primary antibodies for phosphorylated and total AMPK (1:1000 dilution), ABCG1 (1:1000 dilution), ABCA1 (1:1000 dilution), COX-2 (1:1000 dilution), NF- κ B p65 (1:1000 dilution) or CD68 (1:400 dilution) at 4°C for overnight, then horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) at room temperature for 1 h. Signals were developed by enhanced chemiluminescence (Amersham, Piscataway, USA). Images of blots were acquired by use of a digital image processing system (Universal HoodII76S/0608, Bio-Rad, Hercules, CA) and quantified by use of Quantity One (Bio-Rad).

Oil-red O staining

PMA-primed THP-1 cells were sub-cultured in 6-well plates (2.5×10^5 cells/well) equipped with slices, and pre-treated with oxLDL for 24 h, then Compound C for 1 h before co-incubation with HUA and oxLDL for another 24 h. Cells were fixed with paraformaldehyde buffer for 15 min, stained with Oil-red O for 30 min, then counterstained with haematoxylin. Stained cells were viewed by microscopy. The proportion of positive cells was counted.

Statistical analysis

Data are expressed as mean $\pm$ SEM and were analyzed by unpaired Student's t test; comparisons between groups involved one-way ANOVA with Tukey-Kramer post-hoc testing. Statistical analysis involved use of SPSS 17.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant. Figures were obtained by use of GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

HUA treatment induced oxidative stress in PMA-primed THP-1 cells and NAC blocked ROS production

NAC is an antioxidant used to scavenge reactive oxygen species. Our previous study indicated that HUA treatment induced oxidative stress in both hepatocytes and pancreatic β cells [34, 35]. To examine whether HUA treatment also induced oxidative stress in PMA-primed THP-1 cells, cells were sub-cultured in 6-well plates and ROS production was measured by DCFH-DA staining. Treating THP-1 cells with 15 mg/dl uric acid for 120 min strongly increased ROS levels as compared with no treatment or controls; moreover, pretreatment with NAC completely scavenged HUA-increased ROS levels. DCFH-DA-positive cells were increased by 2.02-fold with HUA treatment as compared with controls (Fig. 1). Thus, HUA treatment induced oxidative stress in THP-1 cells.

Fig. 1. Effect of high uric acid (HUA) on reactive oxygen species (ROS) production in phorbol-12-myristate-13-acetate (PMA)-primed THP-1 cells. PMA-primed THP-1 cells were divided into 6 groups as described in Methods and stained with DCFH-DA for fluorescence microscopy (A, magnification $\times 200$), and flow cytometry (B). Quantification of DCFH-DA-positive cells shown as mean $\pm$ SEM from 3 independent experiments; *** $p < 0.001$ vs. vehicle, ### $p < 0.001$ vs. HUA

Fig. 2. (A) HUA treatment time-dependently increased AMPK phosphorylation of PMA-primed THP-1 cells. THP-1 cells were induced with 160 nM PMA for 24 h, then treated with uric acid (15 mg/dl) for various times. Western blot analysis of total and phospho-AMPK level. Data are mean $\pm$ SEM from 3 independent experiments; * $p < 0.05$ vs. 0 min. (B) HUA activates AMPK pathway signaling in THP-1 cells, which is blocked by NAC, an inhibitor of ROS. THP-1 cells were pretreated with NAC (10 or 20 mM; low and high, respectively) for 1 h, co-incubated with 160nM PMA for 24 h, then exposed to HUA (15 mg/dl) for 2 h. Data are mean $\pm$ SEM from 3 independent experiments; * $p < 0.05$, ** $p < 0.01$. ns: not significant.

HUA treatment time-dependent increased phospho-AMPK α (Thr-172) level in PMA-primed THP-1 cells

PMA-primed THP-1 cells were exposed to 15mg/dl uric acid for different times, HUA treatment markedly increased phospho-AMPK α (Thr-172) level at 30, 60, and 120 min (Fig. 2A).

HUA treatment increased phospho-AMPK α (Thr-172) level by inducing oxidative stress in PMA-primed THP-1 cells

To confirm whether HUA treatment activates AMPK by inducing oxidative stress, THP-1 cells were pre-incubated with NAC for 1 h before co-culture with PMA for 24 h, then with 15 mg/dl uric acid for 120 min. Phospho-AMPK α (Thr-172) level was lower with NAC co-culture than HUA treatment alone (Fig. 2B). Thus, HUA treatment increased phospho-AMPK α (Thr-172) level by inducing oxidative stress in THP-1 cells.

HUA treatment inhibited CD68 expression in THP-1 cells during cell differentiation, and Compound C reversed this effect

The AMPK pathway has been suggested to play a key role in regulating the phenotype of monocytes/macrophages [27, 28]. To determine whether HUA treatment impaired CD68 expression by activating the AMPK pathway in THP-1 cells during differentiation, cells were pre-treated with Compound C (10 μ M) for 1 h before 15 mg/dl uric acid was added. As compared with PMA treatment alone, co-treatment with 15 mg/dl uric acid significantly inhibited CD68 expression in THP-1 cells (Fig. 3A); moreover, pretreatment with Compound C completely reversed the HUA-impaired CD68 expression.

To confirm these results, we used anti-human CD68 FITC to label THP-1 cells and determined the expression of CD68 by flow cytometry. As expected, HUA treatment impaired CD68 expression in THP-1 cells (Fig. 3B).

HUA treatment reduced oxLDL uptake in THP-1 macrophage-derived foam cells

To examine relationship of AMPK and oxLDL uptake in THP-1 macrophage-derived foam cells, we used Oil-red O staining to determine oxLDL uptake. OxLDL uptake in foam

Fig. 3. HUA treatment impaired CD68 expression in PMA-primed THP-1 cells by activating AMPK signal pathway. THP-1 cells were pre-incubated with Compound C (10 μ M) for 1 h, then exposed to uric acid (15 mg/dl) for 2 h before co-incubation with PMA (160 nM) for 24 h. Western blot analysis (A) and flow cytometry (B) of CD68 expression in PMA-primed THP-1 cells. Data are mean $\pm$ SEM from 3 independent experiments for western blot analysis and flow cytometry. * p <0.05; ** p <0.01; *** p <0.001; ns: not significant; CC: Compound C. (C) HUA treatment reduced oxLDL uptake in THP-1 macrophage-derived foam cells (magnification $\times$ 400). PMA-primed THP-1 cells were incubated with oxLDL (50 mg/L) for 24 h, then co-incubated with Compound C (10 μ M) and HUA (15 mg/dl) for another 24 h, then stained with Oil-red O and counterstained with haematoxylin. * p <0.05; ** p <0.01; *** p <0.001; ns: not significant.

cells was inhibited by HUA treatment, and Compound C attenuated this effect (Fig. 3C). Thus, HUA-inhibited oxLDL uptake is associated with AMPK activity.

HUA treatment significantly increased the expression of ABCG1 and reversed the oxLDL-reduced ABCG1 expression in PMA-primed THP-1 cells

To assess the effect of HUA treatment on the expression of ABCG1 and ABCA1 induced by oxLDL in PMA-primed THP-1 cells, cells were pretreated with 15 mg/dl uric acid for 30 min and co-incubated with 50 mg/L oxLDL for 24 h. HUA treatment significantly increased the expression of ABCG1 and reversed the oxLDL-reduced ABCG1 expression (Fig. 4). However, the expression of ABCA1 did not differ among controls or HUA-, oxLDL- and HUA+oxLDL-treated cells (data not shown).

Fig. 4. HUA treatment significantly increased the expression of ABCG1 and reversed the oxLDL-reduced ABCG1 expression in PMA-primed THP-1 cells. PMA-primed THP-1 cells were pre-incubated with HUA (15 mg/dl) for 30 min, then co-incubated with oxLDL (50 mg/L) for 24 h. Western blot analysis of the protein expression of ABCG1. Data are mean $\pm$ SEM from 3 independent experiments, *** $p < 0.01$.

Fig. 5. The expression of NF- κ B (p65) and cyclooxygenase 2 (COX-2) was not affected by HUA treatment in THP-1 cells. PMA-primed THP-1 cells were incubated with HUA (15 mg/dl), lipopolysaccharide (LPS; 100 ng/ml), or vehicle for 8 h. Western blot analysis of the protein expression of NF- κ B (p65) and COX-2. Data are mean $\pm$ SEM from 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; ns: not significant.

The expression of NF- κ B (p65) and COX-2 was not affected by HUA treatment in THP-1 cells

Both NF- κ B and COX-2 are the classical pathways regulating the inflammatory response in immune cells [36-40], especially monocytes/macrophages. To determine whether the expression of NF- κ B (p65) and COX-2 was affected by HUA level, PMA-primed THP-1 cells were treated with 15 mg/dl uric acid. The expression of NF- κ B (p65) and COX-2 did not differ with and without HUA treatment (Fig. 5).

Discussion

Increasing research has confirmed that HUA treatment can induce oxidative stress in different kinds of cells, including adipocytes, vascular endothelial cells and renal glomerular mesangial cells, and result in damaged cell function [29-33]. Our previous study showed that HUA treatment induced oxidative stress in both hepatocytes and pancreatic β cells, thereby inducing insulin resistance and inhibiting cell proliferation, respectively [34, 35]. ROS generation is a conservative defense mechanism of innate immune cells, playing a key role in inhibiting the immune response; however, mitochondrial ROS can trigger secretion of pro-inflammatory cytokines [41]. The underlying mechanism by which HUA exposure impairs the immune function is still unknown. In this study, we focused on the generation of ROS after HUA treatment of a human monocytic cell line, THP-1, and found that oxidative stress activated the AMPK pathway, thereby disrupting CD68 expression in THP-1 cells. HUA treatment inhibited oxLDL-induced foam-cell formation in THP-1 cells, which was

blocked by Compound C treatment (Fig. 6). Our data indicate that HUA exposure inhibits monocyte/macrophage function and foam-cell formation by activating the ROS-AMPK signal pathway and blocking CD68 expression.

The negative regulation of the AMPK pathway on phenotype differentiation of monocytes/macrophages has been reported in several studies [27, 28]. However, the relationship between the AMPK pathway and CD68 expression in the phenotype differentiation of monocytes/macrophages is rarely understood. A recent study of animal models of endotoxemia-induced liver injury demonstrated that AICAR, an AMPK activator, or Compound C, an AMPK inhibitor, could attenuate LPS-induced CD68 expression [42]. Our results suggested that HUA exposure can also activate the AMPK pathway and attenuate CD68 expression in cell differentiation, but Compound C can reverse this effect. LPS is a prototypical endotoxin that irritates the immune response by binding to CD14, TLR4, or MD2 receptors in many cell types [43], including monocytes and macrophages. Uric acid needs to enter the cells by the organic anion transporters [44] but not bind to receptors, which in turn causes a histopathological response in cells. This finding may explain the different response of LPS and HUA exposure in cells.

CD68 is a marker of activated macrophages and plays a key role in oxidized LDL uptake, which is related to foam-cell formation [22, 23]. An early study showed that CD68, SR-A, CD36, CLA-1 and LOX-1 are the five major scavenger receptors and suggested that synergistically increased expression of CD36, CLA-1 and CD68 can contribute to the progression to foam cells from macrophages [45]. Our study confirmed that HUA treatment decreased CD68 expression by activating the ROS-AMPK pathway, which might be a protective reaction to ROS production in macrophages, thereby inhibiting the inflammatory phenotype differentiation. However, whether HUA exposure also affects the expression of other scavenger receptors, thereby negatively regulating foam cell formation, still needs further study. In addition, our study was conducted *in vitro*, so further *in vivo* study with a chronic hyperuricemia mouse model may be needed to clarify the association of hyperuricemia and foam cell formation.

Monocytes/macrophages can uptake oxLDL quickly and develop to become foam cells, which are the main component of atherosclerosis lesions. In this study, we found that HUA treatment activated the AMPK pathway and reduced oxLDL uptake by PMA-primed THP-1 cells. This result agreed with AMPK activation inducing cholesterol efflux from macrophage-derived foam cells and alleviating atherosclerosis lesions [46]. This report also showed that AMPK activation can up-regulate the expression of ATP-binding cassette transporters ABCA1 and ABCG1, two major potential cholesterol efflux pathways from macrophages [46]. Simultaneously, we also found that HUA exposure increased the expression of ABCG1. HUA may increase the efflux of cholesterol by regulating ABCG1 expression. The AMPK pathway may be one of the mechanisms responsible for this process. However, the concrete mechanism of this process and whether other transporters are also involved still need further study.

Many clinical studies have suggested that uric acid levels are associated with atherosclerotic disease development. Indeed, many clinical studies show a general or final effect of HUA. Multiple factors that effect atherosclerosis development include inflammation, vascular pathological change, dyslipidemia, hypertension, insulin resistance, obesity and diabetes. We suggested that decreased CD68 expression by HUA in cell differentiation was associated with reduced foam-cell formation *in vitro*, but we have not yet assessed the effect of HUA level on foam-cell formation in mature macrophages *in vivo*. We also deduced that HUA induced decreased CD68 expression in cell differentiation is an early change before foam-cell formation, which also needs further study. Some study suggested that xanthine oxidoreductase and endogenous uric acid production are involved in macrophage foam-cell formation and atherosclerosis development by animal experiments and J774.1 cells [47]. Our experiment explored the effect of exogenous HUA on foam-cell formation in another angle. Further study is needed to confirm the mechanism of endogenous uric acid production and exogenous HUA in atherosclerosis development.

In conclusion, we elucidated the inhibitory effect of HUA exposure on the inflammatory monocyte/macrophage differentiation phenotype and foam cell formation. As shown

Fig. 6. The schematic representation of HUA-impaired CD68 expression and foam-cell formation in human monocytes/macrophages. HUA exposure increases oxidative stress in cells, which activates the AMPK signaling pathway, thereby inhibiting CD68 expression and foam-cell formation. NAC, an antioxidant; Compound C, an AMPK inhibitor.

as Fig. 6, HUA exposure induced oxidative stress and increased ROS levels in human monocytic THP-1 cells, which activated phospho-AMPK α (Thr-172), then inhibited CD68 expression. The process led to disrupted inflammatory phenotype differentiation and foam-cell formation and could contribute to abnormal immune function. Future work may provide additional insights into the involvement of monocytes/macrophages in HUA-induced ROS mechanisms that contribute to the pathological course of hyperuricemia-related diseases.

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Disclosure Statement

No potential conflicts of interest were disclosed.

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