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Effects of niacin and chromium on the expression of ATP-binding cassette transporter A1 and apolipoprotein A-1 genes in HepG2 cells☆

Pilaiwan Siripurkpong, Kesara Na-Bangchang*

Faculty of Allied Health Sciences, Thammasat University, Khong Laung, Pathum Thani 12121, Thailand

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Abstract

The ATP-binding cassette transporter A1 (ABCA1) and apolipoprotein A1 (ApoA-1) are both involved in the regulation of cholesterol efflux from cells. The overexpression of ABCA1 and ApoA-1 genes are associated with increased high-density lipoprotein (HDL) levels. Previous studies have shown that niacin and chromium reduce plasma cholesterol while increasing HDL levels. The aim of the present study was to determine the effects of niacin and chromium on HDL formation by investigating the changes in ABCA1 and ApoA-1 transcription in the human hepatoblastoma cell line (HepG2 cells). Cells were treated with either niacin or chromium, or the combination of both. The expression of ABCA1 and ApoA-1 mRNA was measured by a relative quantitative real-time reverse transcriptase-polymerase chain reaction method. Results showed that niacin at concentrations of 1 and 5 mM significantly increased ABCA1 (1.3–1.7-fold), without affecting ApoA-1 (0.8–1.2-fold), whereas chromium at 3 mM significantly increased both ABCA1 (1.7±0.01-fold) and ApoA-1 (1.5±0.1-fold) transcription when compared to untreated cells. Niacin and chromium cotreatment significantly induced the expression of peroxisome proliferator-activated receptor-α (PPARα) mRNA by approximately 1.3–1.8-fold. It was likely that the increases observed for the ABCA1 transcript may be regulated by the increases in PPARα transcription. A combination of niacin and chromium chloride did not significantly increase (3+1 mM) but instead reduced (1+3 mM) ABCA1 gene expression. In the case of *ApoA* gene, the combination of niacin and chromium chloride at concentrations of 1+3 mM significantly elevated expression; however, this effect was not observed at concentrations of 3+1 mM. When cells were treated with the combination at both concentrations, only slight increases in PPARα mRNA was observed. Niacin, but not chromium, significantly reduced intracellular cholesterol. We hypothesize that the stimulation of *ABCA1* gene expression causes an enhanced cholesterol efflux, perhaps mediated by PPARα pathway(s).
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Keywords: Chromium; Niacin; ATP-binding cassette transporter A1; Apolipoprotein A-1

1. Introduction

Low plasma high-density lipoprotein (HDL) cholesterol levels constitutes an independent risk factor for cardiovascular disease (CVD) [1–4]. As a consequence, a means of increasing HDL has become a recent therapeutic initiative for the treatment of patients considered to be at high risk for CVD [5–7]. HDL is believed to decrease this risk by reversing cholesterol transport, a process by which HDL transports excess cholesterol from peripheral cells, including those in the coronary artery, to the liver for excretion [8–10]. Furthermore, HDL protects low-density lipoprotein (LDL) from oxidation [11] and inhibits the expression of adhesion molecules in endothelial cells, preventing monocyte movement into vessel walls [12]. A major breakthrough in the understanding of the mechanism of reverse cholesterol transport occurred through the discovery that the adenosine-5′-triphosphate (ATP)-binding cassette transporter A1 (ABCA1) transporter is the molecular defect in Tangier disease [13–16]. The low plasma HDL levels seen in Tangier patients is caused by a decreased cellular cholesterol efflux resulting from ABCA1 transporter gene mutations [16]. These studies identified ABCA1 as a transporter that effluxes excess cellular cholesterol to lipid-poor apolipoprotein A1 (ApoA-1) to form nascent HDL [17], which is finally converted to mature α-HDL by esterification

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* Corresponding author. Medical Technology department, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani 12121, Thailand.

E-mail address: kesaratmu@yahoo.com (K. Na-Bangchang).

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of cholesterol to cholesteryl ester by lecithin cholesterol acyltransferase [6,18]. Since hepatic ABCA1 transporter and ApoA-1 level are the major determinants of plasma HDL cholesterol levels, the regulation of ABCA1 gene expression is likely to play a key role in determining intracellular cholesterol concentrations. Analysis of the ABCA1 promoter has revealed several promoter elements, including LXR elements and an E-box, as well as binding motifs for Sp1, Sp3 and AP1 [19,20]. Enhanced expression of the ABCA1 transporter gene increases efflux of intracellular cholesterol to lipid-poor apoA-1 to form pre-β-HDL or nascent HDL [21–24].

Niacin (vitamin B3) was the first lipid lowering drug used to improve patient survival during myocardial infarction. It lowers plasma levels of triglycerides, total cholesterol, LDL while increasing HDL [25–27]. The mechanisms of action have been demonstrated to be via the inhibition of peripheral lipolysis and hepatic very low-density lipoprotein (VLDL) synthesis, the degradation of shunt apolipoprotein B and a decreased ApoA-1 removal [25,27–31]. The effect of niacin on ABCA1 gene expression has been suggested as another mechanism of action. Although niacin has been used to raise HDL, few studies have addressed its cellular mechanism of action on HDL metabolism [27].

Chromium is an essential trace element for both humans and animals. Human studies show that metabolic disorders associated with low chromium intake cause significantly abnormal glucose fluctuations and disturbances in lipid metabolism, which may then lead to development of arteriosclerosis [32–34]. Some researchers suggest that this is a factor responsible for the high frequency of coronary artery disease seen in chromium-deficient individuals [35]. The protective effect of chromium against the development of heart disease is not yet fully understood. In addition, chromium supplementation also has an effect by increasing levels of blood HDL cholesterol in both subjects with Type 2 diabetes mellitus [36] and subjects without diabetes [34,36,37]. However, the cellular mechanism(s) of action of chromium on HDL metabolism is yet to be determined.

In the present study, the effects of niacin and chromium on the expression of ABCA1 or ApoA-1 transcripts, as well as the effects of cotreatment with niacin and chromium, were investigated in the HepG2 hepatic cell line. In light of the findings, peroxisome proliferator-activated receptor-α (PPARα) elements located in various genes that are involved in lipid metabolism and energy homeostasis, including liver X receptor (LXRs) genes, are discussed.

2. Materials and methods

2.1. Cell culture and treatments

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in T-25 tissue culture flasks (Nunc) with 4-ml minimum essential medium Eagle α modification (MEM-α) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 μg/ml penicillin G and 100 μg/ml streptomycin). Cells were grown in a humidified incubator at 37°C under 5% CO_2 atmosphere and passaged every third day. Cells were seeded at density of 4–5×10^4 cells in six-well plates containing 4 ml of standard medium with 10% FBS and grown to 70–80% confluence. Following a starvation period in serum free medium for 24 h, cells were washed with phosphate-buffered saline (PBS) and incubated for 24 and 48 h in medium containing niacin (1, 5 mM, Sigma, St. Louis, MO), chromium chloride ($CrCl_3$: 1 and 3 mM, Fluka, Chemie, Buchs, Germany), a combination of niacin and chromium chloride (1+3 and 3+1 mM) or control medium lacking both niacin and chromium. The concentrations of niacin and chromium chloride used were selected on the basis of previous studies [38,39]. No toxic effects on cell viability were observed as adjudged by thiazolyl blue tetrazolium bromide and trypan blue exclusion assays.

2.2. RNA isolation and amplification using real-time reverse transcriptase-polymerase chain reaction

Total cellular RNA was isolated from 3–4×10^6 HepG2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and were then treated with DNAse (Promega, Madison, WI) to remove residual DNA contamination. Specific primers were synthesized (Operon Biotechnologies GmbH, Germany) as previously described for ABCA1 [40], ApoA-1 [41], PPARα [42], and glyceraldehyde-3-phosphate dehydrogenase, GAPDH [43]. Human ABCA1, ApoA-I, PPARα and GAPDH mRNA were quantified using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) with specific primer sequences (Table 1). cDNA synthesis and PCR amplifications were both carried out in a single tube using the iScript One-step RT-PCR kit with SYBR (Bio-Rad, Hercules, CA). Quantification of gene expression was enabled using Bio-Rad iCycler iQ Real-time PCR Detection System or the Rotor-Gene (RG-3000) system. First-strand cDNA synthesis was performed in a total reaction volume of 50 μl containing 1 U of Moloney Murine Leukemia virus (MMLV) reverse transcriptase at 50°C for 10 min. PCRs were initiated via heat-activation of the iTag antibody-mediated hot start DNA polymerase (95°C for 5 min) to

Table 1
Primers used for real-time PCR analysis

Primer name	Primer sequences (5′→ 3′)	Expected size
ABCA1 forward	GCACTGAGGAAGATGCTGAAA	205 bp
ABCA1 reverse	AGTTCCTGGAAGGTCTTGTTCAC	
ApoA-1 forward	AAGGACCTGGCCACTGTGTA	301 bp
ApoA-1 reverse	TCTCCTCCTGCCACTTCTTC	
GAPDH forward	GAAGGTGAAGGTCGGAGTC	226 bp
GAPDH reverse	GAAGATGGTGATGGGATTTC	
PPARα forward	CTATCATTTGCTGTGGAGATCG	121 bp
PPARα reverse	AAGATATCGTCCGGGTGGTT	

Sequences of forward and reverse primer for each target and expected size of PCR products are shown.

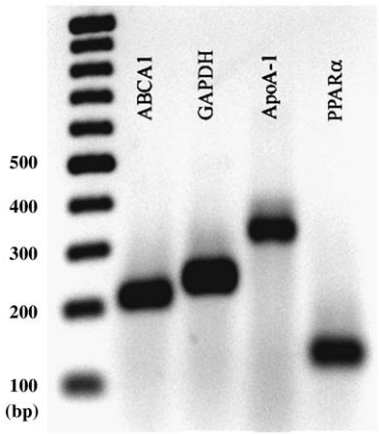

Fig. 1. Ethidium bromide-stained agarose gel separation of specific RT-PCR amplicons of ABCA1, PPARα, ApoA-1 and GAPDH mRNA from HepG2 cells.

release active iTag DNA polymerase and allowing DNA polymerization. PCR was conducted for 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 5 min. Melting curve analysis for PCR products was performed at the final step using 80 steps of 10-s duration each, beginning at 55°C and increasing in temperature by 0.5°C at each step. The amplification was monitored using SYBRGreen as the fluorogenic probe specific for double-stranded DNA. Threshold cycle (Ct), which correlates inversely with target mRNA levels, was measured as the cycle number at which reporter fluorescent emissions increased above threshold levels, using manufacturer's settings. The mRNA levels for ABCA1, ApoA1 and PPARα were normalized with GAPDH mRNA levels. The differences in gene expression between treated and untreated cells were presented as the relative expression ratios (R) or fold changes calculated using the delta-delta method ($R=2^{-\Delta\Delta Ct}$) [44]. RT-PCR products were also fractionated using ethidium bromide-stained agarose gel electrophoresis for qualitative assessments.

2.3. Hepatic cholesterol contents

HepG2 cells were seeded onto 6-well plates in 4 ml MEM-α containing 10% FBS at 37°C, under an atmosphere of 5% CO_2. Cells were starved for 15–24 h once they had grown to 70–80% confluence (~3 days following seeding). The cells were incubated for 48 h in medium containing niacin (1, 3, 5 mM), chromium chloride (3 mM), or control medium, and then washed 1–2 times with PBS. Cells were then incubated with cholesterol (50 μg/ml) in MEM-α medium containing 2% bovine serum albumin for 24 h. After three wash steps with PBS, cells were disrupted using lysis buffer (0.1% sodium dodecyl sulfate, 0.1 M sodium hydroxide). Intracellular lipids were extracted utilizing the Bligh and Dyer Method [45], and total cholesterol content was measured using the CHOD-PAP enzymatic assay (Boehringer Mannheim, Mannheim, Germany). A bicinchoninic acid (BCA) protein assay kit (PIERCE, Biotechnology, Rockford, IL, USA) was used to measure cellular protein contents, thereby permitting results to be normalized for total cellular protein. Cholesterol amounts remaining in HepG2 cells were reported as percentages of control.

Lipid extractions [45] were used for both tissue homogenates and cell suspensions. Briefly, a 1-ml cell suspension was mixed vigorously with 3.75 ml of chloroform/methanol (1:2 v/v) for 10–15 min. Chloroform (1.25 ml) was then added and mixed for 1 min, followed by the addition of 1.25 ml of distilled water and vortexed for another minute. The solutions were then centrifuged at 1000×g for 10 min. The upper phase was discarded, and the lower phase (lipid extract, 1 ml) collected onto a protein disk with a Pasteur pipette. Following evaporation of the lower phase at 95°C, the lipid extract was reconstituted with 100 μl DMSO, incubated with 1 ml of cholesterol reagent (25°C, 10 min), and absorbances measured at OD_{500}.

2.4. Statistical analysis

Statistical significances of all data were evaluated using the Student's t test. P values <.05 were considered statistically significant.

3. Results

3.1. Real-time RT-PCR and primer specificity

RT-PCR experiments yielded a single band of expected size on agarose electrophoreses gels (Fig. 1, Table 1). Additionally, a melting curve analysis revealed single product specific melting temperatures as follows: ABCA1 (83.0°C), ApoA-1 (85.7°C), PPARα (81.0°C), and GAPDH (82.4°C). No primer-dimers were observed during 45 cycles of the real-time PCR amplification protocol. Optimal and identical real-time amplification efficiencies (E) of target genes (ABCA1, ApoA-1, PPARα) and the reference gene (GAPDH) are presumed as E=2.

3.2. Effect of niacin and chromium chloride on ABCA1, ApoA-1 and PPARα gene expression

3.2.1. ABCA1 gene expression

ABCA1 mRNA levels in niacin- and chromium-treated HepG2 cells gradually increased during the 24–48-hour incubation period (data not shown). Niacin and chromium significantly increased ABCA1 gene expression in HepG2 cells by 1.3–1.7-fold when compared to controls. The relative ABCA1 mRNA levels (mean±S.E.M. values) in cells treated with 1 and 5 mM niacin were 1.4±0.4 and 1.5±0.1-fold, respectively (Fig. 2). In 1- and 3-mM chromium-treated cells, relative ABCA1 mRNA levels were 1.5±0.2- and 1.7±0.01-fold, respectively. However, the combination of niacin and chromium at 3 and 1 mM did not significantly alter gene expression, while a combination at concentrations of 1 and 3 mM appeared to reduce ABCA1 mRNA expression levels.

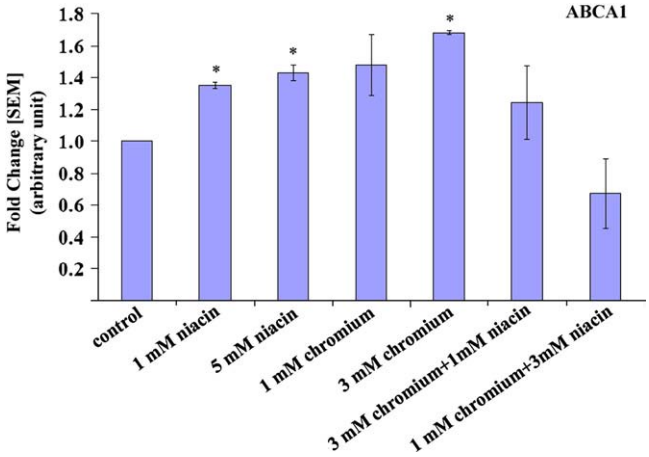

Fig. 2. Effects of niacin, chromium and the combination of both chromium and niacin on the ABCA1 transcription in HepG2 cells. Confluent hepatocytes were incubated for 48 hours in control medium only or in the presence of niacin, chromium chloride or a combination of chromium and niacin. The mRNA levels of ABCA1 were measured using real time RT-PCR as described in Materials and methods and were reported relative to that of GAPDH mRNA as means±S.E.M. (n=3), *P<.05, relative to control.

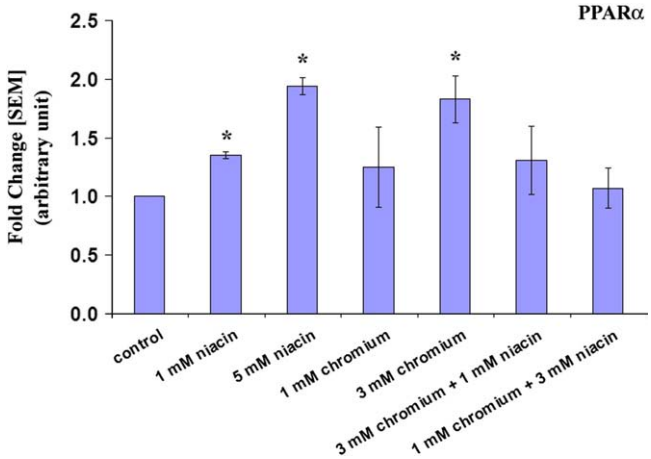

Fig. 4. Effects of niacin, chromium, and the combination of chromium and niacin on PPARα transcription in HepG2 cells. Levels of PARα mRNA were measured by relative quantitative RT-PCR in HepG2 cells kept under control conditions or incubated with niacin, chromium or the combination of chromium and niacin for 48 hours. PPARα mRNA levels were collected for GAPDH (mean±S.E.M., n=3; *=P<.05).

3.2.2. ApoA-1 gene expression

After 48 h, niacin treatment led to statistically insignificant changes to ApoA-1 transcription levels by 0.8±0.1-(1 mM) and 1.2±0.1-fold (5 mM) (Fig. 3). ApoA-1 mRNA levels in chromium-treated HepG2 cells at 1 mM were also not significantly different from that of untreated cells, but significant increases were observed at 3 mM (1.5±0.1-fold) (Fig. 3). The combination of 3 mM chromium chloride and 1 mM niacin significantly stimulated ApoA1 transcription in HepG2 cells (1.4–1.5-fold), but this effect was not observed at the combined concentrations of 1 mM chromium chloride and 3 mM niacin (Fig. 3).

3.2.3. PPARα gene expression

PPARα transcription was studied in order to explore the mechanism of ABCA1 stimulation by niacin and chromium. Niacin significantly induced PPARα transcription by 1.4±0.03-fold at 1 mM and 1.9±0.1-fold at 5 mM (Fig. 4). Chromium chloride at 1 mM produced small (1.3±0.3-fold) increases in PPARα transcription, but significant increases were observed at concentration of 3 mM (1.8±0.2-fold) when compared to untreated HepG2 cells (Fig. 4). Cotreatment with both combinations of niacin and chromium chloride yielded only minor increases in PPARα mRNA (Fig. 4).

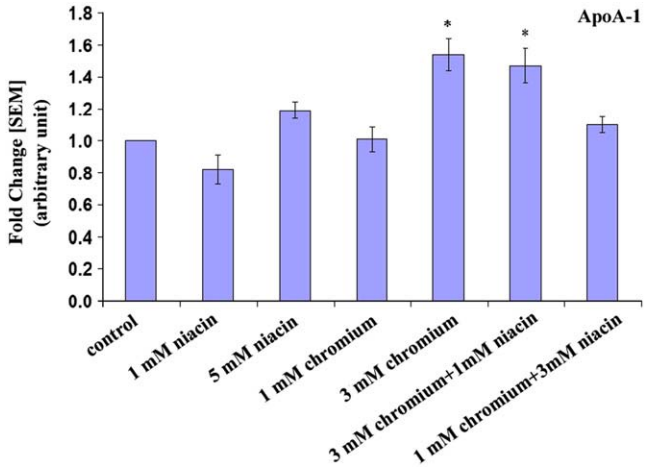

Fig. 3. Effects of niacin, chromium and the combination of chromium and niacin on ApoA-1 transcription in HepG2 cells. Level of ApoA-1 mRNA measured by relative quantitative RT-PCR in HepG2 cells kept under control conditions or incubated with niacin, chromium or the combination of chromium and niacin for 48 hours. ApoA-1 mRNA levels were collected for GAPDH. (mean±S.E.M., n=3; *=P<.05).

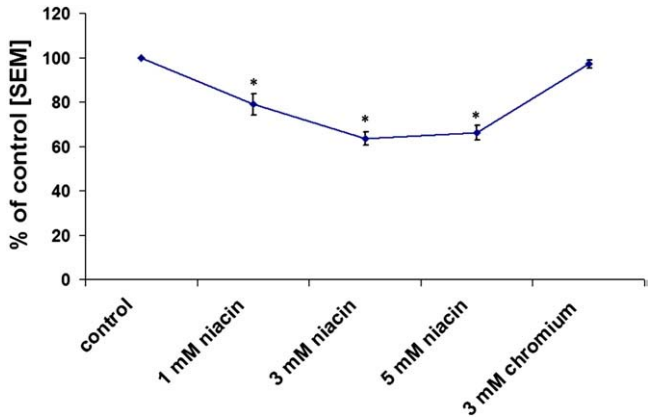

Fig. 5. Effects of niacin and chromium on HepG2 intracellular cholesterol following cholesterol loading. Cells were incubated in control medium only or in the presence of niacin and chromium chloride for 48 h prior to incubation with 50 μg/ml cholesterol for 24 hours. After washing, intracellular lipids were extracted and the total cholesterol content was measured by an enzymatic assay. Results are reported as mean±S.E.M. percent of control (n=2), *P<.05, relative to control.

3.3. Intracellular cholesterol contents

In order to more fully examine the potential effects of niacin and chromium on the stimulation of ABCA1 transcription, the effects of both compounds on cholesterol efflux was investigated by determining the intracellular cholesterol content of HepG2 cells that were first loaded with cholesterol (24 h) and then treated with niacin or chromium (48 h). Niacin significantly reduced the cholesterol content to the levels observed in untreated cells (Fig. 5). It also induced ABCA1 transcription presumably by enhancing cholesterol efflux from HepG2 cells which, in turn, reduced the intracellular cholesterol content. In contrast, the intracellular cholesterol content following chromium chloride exposure were not dissimilar to that of untreated cells.

4. Discussion

The human hepatocyte cell line, HepG2, was used as a model system in the present study to investigate the hepatocellular mechanism of action of niacin and chromium chloride on HDL metabolism. This was done with the perspective that the *ABCA1* gene promoter contains several transcriptional response elements that are potential sites of regulation [19,20,46], some of which may influence constitutive and tissue-specific expression of ABCA1.

4.1. Effect of niacin on ABCA1, ApoA-1, and PPARα transcription

Niacin increased BCA1 transcription by approximately 1.6-fold in HepG2 cells. This observation is in agreement with a previous report for the inducing effect of niacin on ABCA1 transcription (1.5-fold) in HepG2 cells, as well as the monocytic cell line, MM6sr (twofold) [39]. Early human studies indicated that niacin primarily decreased the fractional catabolic rate of ApoA-1 without altering the ApoA-1 synthetic rate [47] and that niacin had no effect on ApoA-1 transcription. Niacin has been reported to increase the accumulation of ApoA-1 in culture medium but exerts no effect on ApoA-1 synthetic processes [30,47]. It was suggested that niacin may influence the removal or the reuptake of HDL by hepatocytes. The selective inhibition of HDL-ApoA-1 uptake, but not HDL-cholesterol esters [30] and HDL-ApoA-1+ApoA-2 particles [48], has also been demonstrated. Together, these studies suggest that niacin, by selective inhibition of hepatic removal/uptake of HDL-ApoA-1 particles, may cause an increased abundance of circulating HDL-ApoA-1 particles in the bloodstream. Indeed, niacin was found to stimulate transcription of ABCA1 and PPARγ in human monocytoid cells [39]. Consistent with this observation, elevated ABCA1 and PPARα transcription were observed in this study following cell exposure to niacin. Induction of ABCA1 by niacin enhances the transport of cellular cholesterol to the outer plasma membrane, upon where it is readily transferred to ApoA-1-containing HDL particles. Thus, an enhanced ABCA1 transcription by niacin counteracts cholesterol efflux by reversing cholesterol transport and HDL formation. Treatment of cells with ligands specific for the LXR and RXR nuclear receptors markedly elevated ABCA1 expression [49]. Ruan et al. [21] demonstrated that PPARα agonists such as bezafibrate stimulated both LXR and ABCA1 gene expression. LXR, existing as heterodimers with RXR, binds to DR-4 sites to elicit the activation of ABCA1 transcription [49,50]. A PPARα response element has been identified in the LXR gene promoter [51], suggesting that PPARα regulates ABCA1 gene expression through the LXR pathway. Therefore, it is likely that increases in PPARα transcription in HepG2 cells are important for the regulation of ABCA1 transcription, mediated by their action on LXR expression and activity. Based on the results for the intracellular cholesterol contents in the present study, we hypothesize that niacin induces the ABCA1 protein over-expression at the HepG2 cell membrane, which mediates the active efflux of cholesterol from cells to ApoA-1 proteins. This would explain why intracellular cholesterol content in niacin-treated HepG2 cells were significantly decreased when compared to untreated cells. However, since niacin was shown to increase the accumulation of ApoA-1 in culture medium in a previous study [30], it is also possible that niacin influences cholesterol efflux by increasing either ABCA1 or ApoA-1 protein levels in culture medium, or by both mechanisms.

4.2. Effect of chromium on ABCA1, ApoA-1, and PPARα transcription

Chromium is an essential mineral and is known to potentiate the action of insulin by enhancing cellular glucose uptake and carbohydrate and lipid metabolism. Trivalent chromium exerts the cholesterol-lowering effect in both human and animal studies [52,53]. In addition, chromium supplementation also increases circulating blood HDL cholesterol levels both in subjects with Type 2 diabetes mellitus and subjects without the disease [36,37]. The cellular mechanism of action of chromium on HDL metabolism remains unclear. Our study has showed that high concentrations of chromium chloride (3 mM) concomitantly increased ABCA1, ApoA-1 and PPARα mRNA transcription in HepG2 cells following 48 h of exposure. In light of this observation, ApoA-1 and ABCA1, both of which are involved in the reverse cholesterol transport and HDL formation, may be possible factors associated with the increases in HDL levels, and the transcription of ABCA1 and ApoA-1 genes could be regulated by PPARα. The transcriptional activity of ApoA-1 promoter is suppressed by dextrose and stimulated by insulin in a dose-dependent fashion [54]. Chromium has an insulinomimetic action and possibly increases ApoA-1 transcript by either direct or indirect mechanisms via

PPARα or insulin responsive elements on the ApoA-1 promoter [55].

Since chromium induced the over-expression of ABCA1 and ApoA-1 transcription, the intracellular cholesterol content in chromium-treated HepG2 cells should be significantly reduced compared to that of untreated cells. However, the results in our present study do not support this. Under the experimental conditions, we observed that chromium-treated HepG2 cells were more difficult to harvest by trypsin and were also somewhat refractory to disruption using lysis buffer. This may be a consequence of the more rigorous composition of extracellular matrix proteins surrounding the cell membranes of chromium-treated cells in contrast to the untreated or niacin-treated cells. Chromium may also induce other mechanistic pathways that lead to increases in the cholesterol synthesis intracellularly [56], resulting in the unchanged intracellular cholesterol contents which were observed in these cells despite the induction of ABCA1 and ApoA-1.

4.3. Effect of the combination of niacin and chromium on ABCA1, ApoA-1 and PPARα transcription

Inorganic chromium is poorly absorbed in the human gut and only 0.4–2.0% of chromium intake as chromium chloride passes into the gastrointestinal tract [57]. The combination of chromium and niacin as a chromium (III) nicotinic acid complex has been shown to increase gut absorption in rats [58]. Several studies have shown that niacin and chromium exert combined effects. Niacin and chromium decrease total plasma levels of cholesterol and triglycerides, and increase HDL cholesterol. The chromium (III) nicotinic acid complex also significantly decreases total cholesterol and lipid levels in serum [59]. Moreover, a double-blinded crossover study revealed that a nicotinic acid-complexed form of trivalent chromium administered at 200 μg/day lowered fasting total and LDL cholesterol, triglycerides, glucose concentrations, and 90-min postprandial glucose levels in individuals with type 2 diabetes. However, the authors stated that these changes were not statistically significant [60]. Our results show that the combination of 3 mM chromium chloride and 1 mM niacin produced a less pronounced effect on ABCA1 mRNA transcription than chromium (3 mM) or niacin (5 mM) alone, while it significantly increased ApoA-1 mRNA transcription similar to that observed with 3 mM chromium chloride treatment. Paradoxically, ABCA1 transcripts of cells treated with the combination of 1 mM chromium and 3 mM niacin produced the opposite results to those obtained with 3 mM chromium chloride or 5 mM niacin treatment alone (Figs. 2 and 3). These results indicate that the combination of chromium and niacin does not exert synergistic effects on the ABCA1 and ApoA-1 transcripts. The mechanism underlying the effects of the combination of chromium and niacin remain unclear and required further clarification.

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