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Brief communication (Original)

Downregulation in the mRNA expression of nuclear hormone receptor liver-X-receptor alpha (LXR-α) by TNF-α is abolished by the antioxidant kaempferol, but not ascorbic acid, in human hepatocarcinoma HepG2 cells

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Background: Liver - X - Receptor alpha (LXR-α) plays an essential role in cholesterol metabolism, bile acids, and lipid synthesis. The proinflammatory cytokine TNF-α has been shown to downregulate the expression of LXR-α.
Objectives: We investigated the effects of antioxidants kaempferol and ascorbic acid on LXR-α mRNA expression in TNF-α stimulated HepG2 cells.
Methods: After the cells have reached 60% to 70% confluence, they were treated with 20 ng/ml of TNF-α for 24 hours prior to the stimulation with different concentrations of kaempferol (1 μM, 5 μM, 10 μM, and 20 μM) or ascorbic acid (15 μM, 150 μM, and 1500 μM) for another 24 hours. Total RNA was isolated using TRI Reagent LS (Molecular Research Centre). Reverse transcription quantitative real-time PCR was then conducted to determine the mRNA expression of LXR- α after their respective treatments.
Results: Kaempferol was able to relieve the downregulatory action of TNF-α on LXR-α in a dose-dependent manner. The highest dose of kaempferol (20 μM) almost abolished the action of TNF-α by restoring the levels of LXR-α to its basal levels in HepG2 cells. Twenty μM of kaempferol alone did not produce any significant effect on LXR-α mRNA expression. In contrast, ascorbic acid was unable to counteract the action of TNF-α. In contrast to kaempferol, the highest dose of ascorbic acid (1500 μM) suppressed the expression of LXR-α mRNA in TNF-α stimulated HepG2 cells to 0.571-fold compared with no treatment. In the presence of 1500 μM of ascorbic acid alone, LXR-α mRNA expression was also significantly suppressed to 0.320-fold.
Conclusion: This study demonstrated that kaempferol, but not ascorbic acid, was able to alleviate the TNF-α downregulatory effects on LXR-α mRNA in HepG2 cells. Kaempferol can be a better anti-inflammatory agent than ascorbic acid.

Keywords: Antioxidants, ascorbic acid, cytokine, expression, kaempferol, LXR-α, TNF-α

Liver X Receptors (LXRs) are nuclear transcription factors involved in cholesterol, bile acids, fatty acids, and lipid metabolism [1]. Studies have shown that LXRs also modulate carbohydrate metabolism and inflammatory processes [2, 3]. LXRs exist in two isoforms, LXR-α (NR1H3) and LXR-β (NR1H2). LXR-α is expressed abundantly in metabolically active tissues like liver, kidney, and adipose tissue, whereas LXR-β is ubiquitously found [2]. LXR-α has been implicated as a main regulator of hepatic lipid metabolism [2].

TNF-α is a multifunctional cytokine synthesized by immune cells such as monocytes and macrophages. It is involved in various cellular processes and diseases especially inflammation and cancers. Low levels of TNF-α can stimulate our immune system, but when it is present at high levels for long term, it causes deleterious effects to the body. TNF-α has been shown to interfere with lipid homeostasis and its signaling is suggested to be proatherogenic [4, 5].

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Kaempferol is a polyphenol flavonoid ubiquitously found in vascular plants, while ascorbic acid is a water-soluble micronutrient available in fruits and vegetables. Both are well-known antioxidants that have been shown to be anti-inflammatory agents. Indeed, kaempferol was able to suppress the proinflammatory cytokine, interleukin-1β-induced MUC5AC gene expression in inflammation [6]. Ascorbic acid is able to function in the maintenance of proper lipoprotein metabolism as a result of its anti-oxidative property [7]. Owing to the evidences above, it is therefore hypothesized that both kaempferol and ascorbic acid can relieve the inflammatory effects introduced by TNF-α.

Thus, the objectives of this study were to investigate the effect of kaempferol and ascorbic acid on LXR-α mRNA expression in TNF-α stimulated HepG2, and to observe whether both the antioxidants were able to alleviate inflammatory responses.

Materials and methods

Cell line and culture

The human hepatocarcinoma cell line, HepG2 cells, was a kind gift from Dr. Tan Mei Lan and was grown in Minimum Essential Medium with Earle's salts medium supplemented with 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM of non-essential amino acids, 1.5 μg/ml sodium bicarbonate, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) in a 5% CO_2 incubator maintained at 37°C. All reagents were obtained from Invitrogen, USA.

Treatment of HepG2 Cells with TNF-á and kaempferol or ascorbic acid

HepG2 cells were cultured in 25 cm^2 tissue culture flasks and allowed to grow until they achieved about 60% to 70% confluence. The medium was then discarded and the cells were washed twice with phosphate buffered saline. For the control sample, the cells were treated with fresh MEM and 0.5% (v/v) FBS, without any cytokine added. For the experimental samples, 20 ng/ml of TNF-α, 0.5% (v/v) FBS and fresh medium were added to treat samples prior to the addition of kaempferol (Sigma Aldrich, USA) or ascorbic acid (Fisher Scientific, UK). The cells were then incubated in incubator of 5% (v/v) CO_2 at 37°C for 24 hours. Different concentrations of kaempferol (1, 5, 10, and 20 μM) or ascorbic acid (15, 150, and 1500 μM) were subsequently added to the TNF-α-pretreated samples. One other flask of HepG2 cells are treated with only 20 μM kaempferol or 1500 μM of ascorbic acid. The cells were then incubated in 5% CO_2 at 37°C for another 24 hours. Upon the completion of the incubation period, the total cellular RNA was extracted using TRI Reagent LS (Molecular Research Centre, Singapore).

Reverse transcription quantitative real-time polymerase chain reaction (RT–qPCR)

RT–qPCR was performed using Quantifast SYBR Green RT-PCR (Qiagen, Germany) according to manufacturer's instructions on a MyIQ iCycler (Bio-Rad, USA) using the protocol which consisted of 1 cycle of 10 minutes at 50°C (for cDNA synthesis), followed by 30 cycles of denaturing at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and primer extension at 72°C for 30 seconds. Melt-curve analysis was performed as follows: 1 minute at 55°C, followed by 10 seconds at 55°C with an incremental 0.5°C for 90 cycles. Primer sequences used to amplify the human *Lxr-α* are as follows: 5′ -TCAGGCGGATC TGTTCTTCT-3′ (*Lxr-α* reverse), 5′ -CGGGCTTC CACTACAATGTT-3′ (*Lxr-α* forward), which produced a 213-bp RT-PCR product. β-actin primers were used as an internal control. The primer sequences for human β-actin were as follows: 5′ -CGTACCAC TGGCATCGTGAT-3′ (forward), 5′ -CCATCTCT TGCTCGAAGTTC-3′ (reverse), which yielded a 280-bp RT-PCR product.

Statistical analysis

An online GraphPad Software (GraphPad Software, La Jolla, CA) was utilized for statistical analysis. Statistical significance was assessed to determine expression of LXR-α of treated HepG2 cells compared to control (untreated HepG2 cells) using a two-tailed, unpaired, Student's *t* test. The criterion for statistical significance was $p < 0.05$.

Results

The expression of LXR-α mRNA increased gradually after treatment with an increasing dose of kaempferol (**Figure 1A**). LXR-α expression was reduced to 0.648-fold when stimulated with 20 ng/ml TNF-α only. As for treatment with TNF-α and 1 μM of kaempferol, the gene expression was further suppressed to 0.478-fold. With increasing dosage of kaempferol (5, 10, and 20 μM) in TNF-α stimulated

cell, LXR-α expression was increased accordingly to 0.719-, 0.889-, and 0.927-fold. Cells treated with only 20 μM kaempferol showed a slightly higher LXR-α expression, which is 1.048-fold, compared with the untreated cells.

On the other hand, the expression of LXR-α mRNA decreased gradually after the treatment with ascorbic acid. As shown in **Figure 1B**, LXR-α expression was reduced to 0.896-fold when treated with only TNF-α. Stimulation with TNF-α and different doses (15, 150, and 1500 μM) of ascorbic acid further reduced LXR-α expression to 0.767-, 0.694-, and 0.571-fold, respectively. As for treatment with 1500 μM of ascorbic acid alone, LXR-α expression reduced significantly to 0.320-fold.

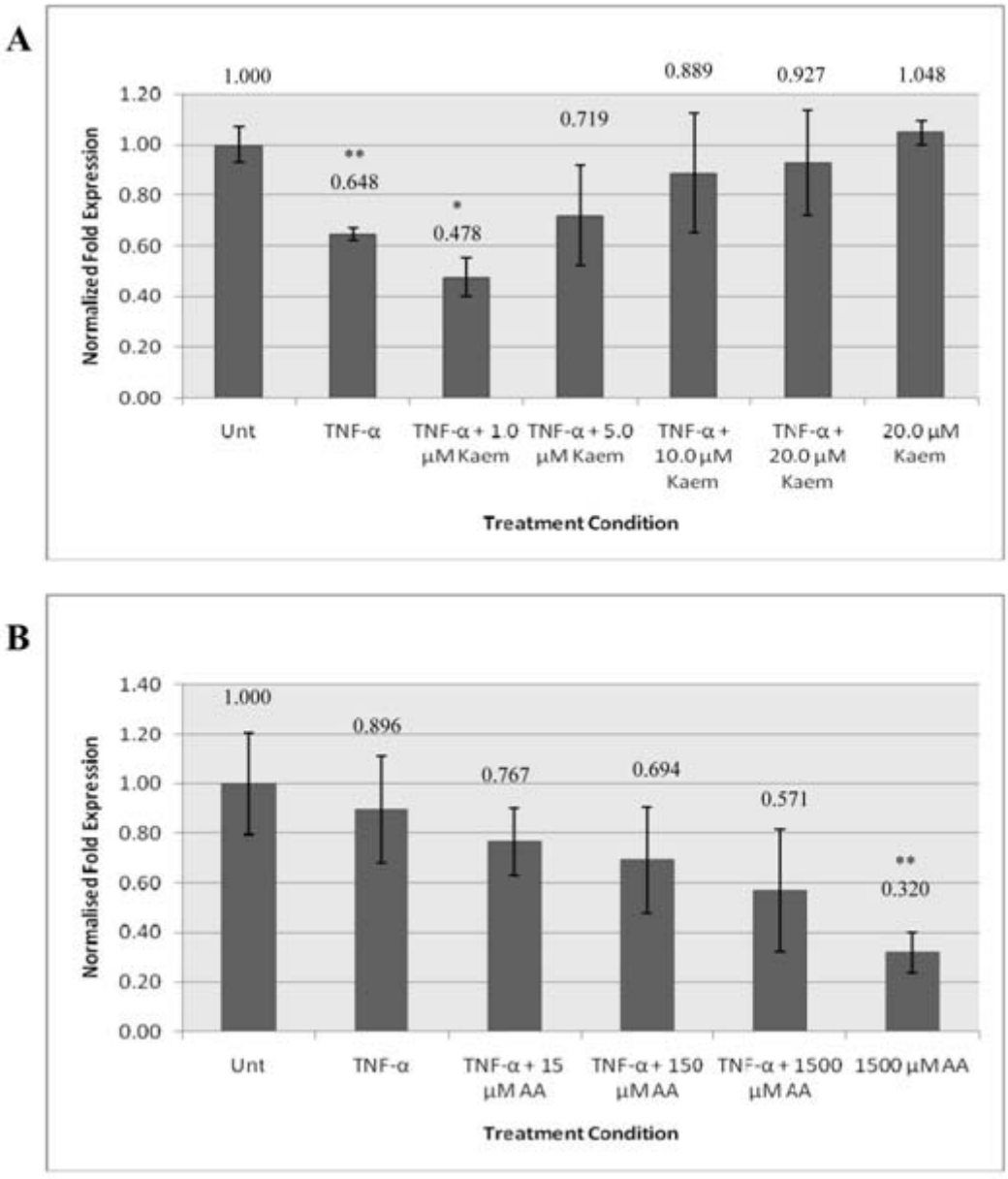

Figure 1. Quantitative expression profile of LXR-α in HepG2 cells stimulated with TNF-α and different doses of (**A**) kaempferol and (**B**) ascorbic acid. The value stated above each bar showed the fold changes in value of LXR-α mRNA expression (normalized to β-actin) and relative to control (untreated HepG2 cells), which was assigned as 1.000. Error bars indicates standard deviation. *$p < 0.05$ and **$p < 0.01$ represent statistical significant change as compared with control (untreated HepG2 cells).

Discussion

Inflammation is the first body response to harmful stimuli such as infection and injury. Functions of inflammation are to resolve infection and to repair the damage as to achieve body homeostasis [8]. TNF-α is cytokine produced in the early phase of inflammation and it regulates the production of other cytokines. It is the main regulator of inflammatory process and involved in the pathogenesis and development of atherosclerosis, myocardial ischemia and other diseases. Because of its crucial role, TNF-α has become a therapeutic target in inflammatory diseases. In this study, cells which were subjected to 20 ng/ml of TNF-α treatment showed reduction in LXR-α mRNA expression. This is supported by studies that also showed that TNF-α decreases the expression of LXR-α mRNA in different cell lines [9, 10].

Kaempferol is one of the main flavonols in our diet. In this study, after the initial TNF-α treatment different concentration of kaempferol (1, 5, 10, and 20 μM) were added into the cell culture for 24-hour incubation. As shown in **Figure 1A**, kaempferol was able to relieve the inflammatory action brought by TNF-α. Studies have shown that kaempferol was able to lower the reactive oxygen species level by increasing the cellular reductant, glutathione in aged rat gingival tissues [11], and reduced the activation of NF-κB [12, 13].

Hypothetically, ascorbic acid should be able to alleviate the action of TNF-α as it can inhibit the NF-κB activation induced by TNF-α by interfering with IêBα phosphorylation [14]. Though, the methodology used in this particular study was different as they first treated the cells with ascorbic acid and then TNF-α. In contrast, it has been reported in another study that ascorbic acid enhanced TNF-α mediated NF-κB activation [15]. According to Kaul and Baba, ascorbic acid was able to induce LXR-α mRNA to 3.5-fold in mononuclear cells. It is proposed here that ascorbic acid have a different effect on LXR-α gene expression in a tissue-specific manner [16].

On the other hand, there are data showing ascorbic acid decreased certain gene expression. Ascorbic acid was proven to inhibit *PMP22* gene expression, which is implicated in Charcot–Marie–Tooth (CMT) syndrome, by reducing intracellular cyclic adenosine monophosphate (cAMP) level [17]. It has been shown to suppress the expression of PPAR-á and PPAR-γ in human mononuclear cells [16]. These inconsistencies in result revealed that the action of ascorbic acid is complicated, and further studies on the pathways involved would be interesting. Furthermore, ascorbic acid has been proven to increase LCAT activity, which is involved in reverse cholesterol transport (RCT) and is responsible for esterification of extrahepatic free cholesterol and their sequestration into HDL [18]. In addition, it is also reported that ascorbic acid increased LDL-R expression, which in turn enhances LDL cholesterol catabolism and reduces serum cholesterol level [19]. Because oxysterol levels are a relative indicator of cholesterol level, low cholesterol level suggests a low oxysterol level. Since oxysterol is a known ligand for LXR-α gene expression [20], low levels of oxysterol will in turn, lower LXR-α mRNA expression. However, the precise mechanism remains to be determined.

Conclusion

This study showed that kaempferol, but not ascorbic acid was able to alleviate the TNF-α downregulatory effects on LXR-α mRNA expression in human hepatocarcinoma HepG2 cells. This suggests that kaempferol is a better anti-inflammatory agent than ascorbic acid.

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