Caffeine is a stimulant LDLR-mediated cholesterol clearance is improved by *SREBP2*-induced hepatic *PCSK9* expression

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Fasolato E. Caffeine is a stimulant LDLR-mediated cholesterol clearance is improved by SREBP2-induced hepatic *PCSK9* expression. Clin Cardiol J. 2022; 6(2):13-14.

ABSTRACT

Caffeine (CF) appears to lower the risk of Cardiovascular Disease (CVD). The process by which this occurs, however, has yet to be discovered. The influence of CF on the expression of two genuine regulators of circulating Low-density Lipoprotein Cholesterol (LDLc) levels, the Proprotein Convertase Subtilisin/Kexin type 9 (*PCSK9*) and the Low-density Lipoprotein Receptor (LDLR), was studied in this study (LDLR). Following the discovery that CF decreased circulating *PCSK9* levels while increasing hepatic LDLR expression, further CF-derived analogues with higher *PCSK9* inhibitory potency than CF were created. The effect of CF on decreasing *PCSK9* was later validated in a group of healthy individuals. We show that CF increases hepatic Endoplasmic Reticulum (ER) Ca^{2+} levels, which inhibits transcriptional activation of the Sterol Regulatory Element-binding Protein 2 (*SREBP2*), which regulates *PCSK9*, resulting in increased LDLR expression and LDLc clearance. Our findings reveal ER Ca^{2+} as a master regulator of cholesterol metabolism and a mechanism through which CF may protect against CVD.

Key Words: *Cardiovascular disease; Low-density lipoprotein receptor; Sterol regulatory element-binding protein 2*

INTRODUCTION

LDLC (Low-Density Lipoprotein Cholesterol) levels in the blood are strongly connected to the development of Cardiovascular Disease (CVD). Despite the approval of various LDLc-lowering medicines, many patients are unable to achieve their LDLc-lowering goals due to intolerance, side effects, or simply the high cost of pharmaceuticals. The Endoplasmic Reticulum (ER)-resident transcription factor Sterol Regulatory Element-binding Protein 2 (*SREBP2*) is an essential regulator of LDLc. Reduced intracellular cholesterol and the decrease of ER Ca^{2+} activate *SREBP2*, causing it to translocate to the nucleus and induce cholesterol regulating genes such *PCSK9, LDLR,* and HMG-CoA reductase (HMGR). *PCSK9* inhibits the ability of metabolically active organs, such as the liver, to remove excess LDLc from the bloodstream [1]. Anti-*PCSK9* antibodies, based on these important discoveries, are now accessible to individuals at high risk of CVD, resulting in a 60%-70% reduction in LDLc levels6. Anti-*PCSK9* antibodies are effective, but their high cost and/or requirement for subcutaneous delivery limits their access to patients globally. In order to produce more cost-effective medicines, greater research into the molecular mechanisms that affect the production and secretion of *PCSK9* from hepatocytes is required.

Caffeine (CF), also known as 1,3,7-trimethylxanthine, is a central nervous system stimulant found in a variety of plants and is usually found in coffee and tea. The majority of published literature shows that the average adult caffeine drinker consumes between 400 mg and 600 mg of caffeine per day, and organisations such as Health Canada and the Food and Drug Administration have concluded that such doses are not linked to toxicity, cardiovascular effects, bone status, calcium imbalance, behaviour, cancer incidence, or effects on male fertility. On the contrary, mounting data suggests that moderate to high levels of CF (>600 mg) eaten daily in the form of non-alcoholic beverages are linked to a lower risk of cardiovascular disease [2]. Although biochemical investigations have demonstrated that CF elevates intracellular Ca^{2+} levels and causes vascular endothelium vasodilation via nitric oxide release, a biological activity known to be cardioprotective, molecular mechanisms supporting clinical proof are still absent.

IN HEPATOCYTES, CF INHIBITS PCSK9 EXPRESSION AND SECRETION

To begin our research, we treated cultured immortalised hepatocytes known to express and secrete *PCSK913*, such as *HuH7* and *HepG2* cells, as well as primary mouse and human hepatocytes (PMH and PHH, respectively) with CF for 24 hours and measured *PCSK9* expression using immunoblots and real-time PCR. These preliminary studies suggested that CF decreased *PCSK9* protein and mRNA transcript levels. CF also reduced *PCSK9* expression induced by thapsigargin, a *SERCA* pump antagonist and well-known ER stress-inducing drug. Because sterol depletion is a well-known activator of *SREBP2* activation, cells were additionally given CF in the presence and absence of *U18666A* (U18), a pharmacological drug that depletes intracellular sterols. CF inhibited U18-induced *PCSK9* expression in the same way that TG did [3].

Importantly, CF inhibited *PCSK9* secretion in *HuH7* and *HepG2* cultured hepatocytes, as well as *PMH* and *PHH*. By assessing levels of recombinant *PCSK9* in the presence and absence of CF, control studies showed that CF did not interfere with the ELISA. To confirm that CF was not influencing global protein production, a Coomassie stain of electrophoretically resolved medium taken from these cells was employed. After then, *HepG2* cells were given an increasing dose of CF. In the presence of ActD, CF fails to suppress *PCSK9* mRNA and secreted protein, implying that CF has a transcription-dependent effect on *PCSK9*. CF also failed to suppress *PCSK9* secretion in cells transfected with a CMV-driven *PCSK9* vector, corroborating these findings. These findings show that CF can significantly reduce PCSK9 expression at the mRNA and protein levels in a variety of cultured hepatocyte cell types at nanomolar concentrations, implying that the suppression occurs at the transcriptional level [4].

IN HEPATOCYTES, CF INHIBITS SREBP2 ACTIVITY

ER stress, specifically depletion of ER Ca^{2+}, enhances the activation of SREBP2 and the production of *PCSK9*, as our research group has previously showed. As a result, we investigated the impact of CF on TG-induced *SREBP2* activation. We found that CF inhibited the expression of *SREBP2* in PMHs and PHHs, as well as in *HepG2* cells, which is consistent with earlier research. CF also blocked the expression of a downstream target of *SREBP2* transcriptional activity in PMHs, *HMGR*, as well as *SREBP1*, the isoform known to regulate fatty acid synthesis. In the absence of TG, the effect of CF on the expression of hepatocyte nuclear factor 1a, a liver-expressed transcription factor likewise known to influence *PCSK9* expression, was investigated, but no significant difference was found. In HuH cells transfected with a plasmid encoding GFP driven by the sterol regulatory element, *SREBP2* activity was evaluated at the protein level. In the presence and absence of TG, CF inhibited the nuclear/activated isoform of SREBP2 (nSREBP2; 60 kDa) and the expression of SRE-driven GFP, which was

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Received: 03-March-2022, Manuscript No. PULCJ-22-4615; Editor assigned: 05-March-2022, PreQC No. PULCJ-22-4615(PQ); Reviewed: 11-March-2022, QC No. PULCJ-22-4615(Q); Revised: 13-March-2022, Manuscript No. PULCJ-22-4615(R); Published: 26-March-2022, DOI: 10.37532/pulcj.22.6(2).13-14

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consistent with the real-time PCR results. Immunofluorescent labelling was used to visualise GFP expression, which was then quantified using ImageJ software.

SREBP2 immunofluorescence labelling in cells treated with TG in the presence and absence of CF revealed that CF inhibited *SREBP2* re-localization from the perinuclear area to the nucleus. Given *SREBP2*'s well-known function in *PCSK9* transcriptional control, our findings suggest that CF suppresses *PCSK9* expression and secretion via inhibiting de novo synthesis. Following that, PMHs were isolated from Wildtype (WT) and Ampk1/ mice. *PCSK9* expression and secretion were reduced in these hepatocytes after treatment with CF, indicating that AMPK is not directly implicated in CF-mediated *PCSK9* suppression. *CDN1163* (CDN), a pharmacologic drug known to raise ER Ca^{2+} levels by triggering SERCA pump activation, produced a comparable outcome in hepatocytes.

PCSK9 EXPRESSION AND SECRETION ARE INFLUENCED BY ER Ca^{2+}

CF's potential to enhance intracellular Ca^{2+} levels is well-studied among its various intracellular effects. We hypothesised that (a) CF may raise ER Ca^{2+} levels, and (b) additional drugs known to enhance ER Ca^{2+} levels may also prevent *SREBP2* activation and *PCSK9* expression, based on our earlier findings that ER Ca^{2+} depletion increases *SREBP2* activation. To test this idea, we used the high-affinity fluorescent Ca^{2+} indicator Fura-2-AM to look at cytosolic Ca^{2+} levels in CF-treated cells. In immortalised hepatocytes, CF dramatically raised cytosolic Ca^{2+} levels, which was consistent with prior research. *D1ER*, a genetically encoded ER-resident fluorescence resonance energy transfer (FRET)-based calreticulin chameleon Ca^{2+} sensor that increases in fluorescence intensity upon Ca^{2+} binding, was then used to monitor ER Ca^{2+} levels in cells transfected with it. Mag-Fluo-4, a low-affinity Ca^{2+} indicator, was also used to measure ER Ca^{2+} and fluorescence intensity changes in response to Ca^{2+} binding [5]. Using a fluorescent spectrophotometer and a fluorescent microscope, the fluorescence intensity of cells treated with CF and control agents, TG and CDN, was measured and visualised. We discovered that, in addition to elevated cytosolic Ca^{2+} levels, CF also enhanced ER Ca^{2+} levels. The control agent CDN increased ER Ca^{2+} levels, as expected, but TG decreased ER Ca^{2+} levels. The high-affinity Ca^{2+} dye Fura-2-AM was used to evaluate ER Ca^{2+} concentration indirectly. HuH7 cells were pretreated with CF for 24 hours before being exposed to a high dosage of TG, which causes ER Ca^{2+} to be lost spontaneously. When cells were exposed to TG after being pretreated with CF, they showed enhanced ER Ca^{2+} efflux compared to cells treated with the vehicle control. We also discovered that CF promoted and TG inhibited the protein production of calnexin, an ER-resident protein with a high Ca^{2+} binding capability [6].

ELISAs were used to measure secreted PCSK9 levels in the medium of cells treated with Ca^{2+}-modulating drugs. We found that high-dose ryanodine, CDN, and 2APB inhibited PCSK9 secretion, which was consistent with real-time PCR results. PCSK9 secretion was likewise prevented by overexpression of calnexin and loss-of-function ryanodine receptor mutants (RyR2E4872A and RyR2A4860G), which have previously been demonstrated to raise ER Ca^{2+} levels. We found that, in contrast to its influence on PCSK9 mRNA transcript levels, TG inhibited *PCSK9* secretion, which is consistent with our earlier findings [7]. Sterol deprivation via treatment with U18, which hasn't been shown to influence ER Ca^{2+} levels, resulted in enhanced *PCSK9* secretion, which was in line with our prior findings. Finally, tests were performed with HepG2 cells cultured in Ca^{2+}-deficient media for 48 hours to establish that CF prevented PCSK9 secretion in a Ca^{2+} dependent manner. We previously showed that this therapy causes significant ER stress, which accounts for the observed decrease in secreted PCSK9 levels in the absence of CF. Importantly, these findings show that CF has no effect on *PCSK9* production in cells that have been depleted of Ca^{2+}. Overall, these findings show that ER Ca^{2+} levels influence not only the production of ER stress indicators, but also the regulation of *PCSK9* and *SREBP2* [8].

DISCUSSION

Others have looked into the impact of CF on the vascular system and CVD in the past. Because CF is commonly consumed in the form of beverages with variable doses and often mixed with adulterants such as dairy and sugar products, the results of such trials can be difficult to understand and often change. A new meta-analysis gives a comprehensive overview of the current body of knowledge on the effects of CF intake on cardiovascular outcomes, including total CVD. Surprisingly, the majority of research looked at, which included tens of thousands to hundreds of thousands of individuals, found that consuming CF reduced the risk of cardiovascular disease. The inhibition of adenosine receptors, GABA receptors, and phosphodiesterase enzymes, as well as generating intracellular Ca^{2+} transients through increasing RyR-mediated Calcium-induced Calcium Release (CICR)11, are all established molecular targets for CF. Although the above interactions do not directly support our finding that CF raised ER Ca^{2+} levels, CF is also known to decrease ER Ca^{2+} release by inhibiting the IP3-receptor40,41. CF has also been reported to bind to hepatic RyR, presumably blocking RyR-mediated Ca^{2+} release42. The determination of the molecular processes relating to CF's protective effect on the vascular system is difficult given the wide range of targets known to interact with it [9]. The ER functions as a vital and dynamic Ca^{2+} reserve, capable of extruding Ca^{2+} for signalling and/or excitatory reasons, as well as eliminating excess cytosolic Ca^{2+} after periods of excitation. The quantity of ER-resident low-affinity/high-capacity Ca^{2+}-binding proteins determines the ER's Ca^{2+} sequestering capability, which greatly exceeds that of the cytoplasm. Chaperone function is also aided by the interaction of Ca^{2+} with these proteins. Chaperones lose their folding capacity when ER Ca^{2+} is depleted, and misfolded polypeptides accumulate in the ER.

The UPR is then activated in order to restore ER folding capacity and Ca^{2+} levels by increasing the quantity of ER-resident chaperones. Given the observed reduction in UPR marker expression by drugs that activate SERCA and increase ER Ca^{2+} influx or those capable of preventing leakage from either IP3R or RyR, boosting ER Ca^{2+} levels appears to have a net beneficial effect on ER homeostasis. We also discovered that CF protected cultured hepatocytes against TG-induced ER stress and lowered the expression of a number of ER chaperones in the livers of mice, which is consistent with our findings. PCSK9 promotes the beginning and progression of CVD by enhancing LDLR degradation, which is one of the most difficult and costly health care issues that society faces today. Understanding the regulatory mechanisms that regulate *PCSK9* production and secretion from hepatocytes could contribute in the development of anti-PCSK9 medicines that are less expensive than those currently available. Overall, our findings support a concept in which small compounds such as CF, which can raise ER Ca^{2+} levels, can inhibit SREBP2 activation by improving GRP78 chaperone activity and binding ability [10].

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