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Identification and characterization of β-sitosterol target proteins

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ABSTRACT

β-Sitosterol is the most abundant plant sterol in the human diet. It is also the major component of several traditional medicines, including saw palmetto and devil's claw. Although β-sitosterol is effective against enlarged prostate in human clinical trials and has anti-cancer and anti-inflammatory activities, the mechanisms of action are poorly understood. Here, we report the identification of two new binding proteins for β-sitosterol that may underlie its beneficial effects.

Phytosterols are a group of steroids produced by plants. They are structurally and functionally related to cholesterol and comprise a major component of the human diet.[1,2] Among them, β-sitosterol (24-ethylcholesterol) is one of the most abundant dietary phytosterols present in many beans, nuts, and seeds (Fig. 1).[3–5] It is also an important constituent of saw palmetto, devil's claw, stinging nettle and several other natural remedies.[6–8] β-Sitosterol consumption has been reported to decrease blood cholesterol levels by preventing its intestinal absorption.[9,10] It also has been shown to have anti-inflammatory and analgesic properties in various animal models.[11–14] Additionally, in both animal models and human clinical trials, β-sitosterol has demonstrated a significant effect on reducing the symptoms of benign prostatic hyperplasia.[15,16] β-Sitosterol intake may also be partially responsible for the decreased incidence of prostate, colon and breast cancers among vegetarians and men and women in Asian countries who consume much larger amounts of β-sitosterol than most Westerners.[5,17] In support of this hypothesis, β-sitosterol exhibits growth inhibitory and cytotoxic effects against a range of cancer cell lines.[7,18–20] However, the precise molecular mechanisms underlying its health promoting effects remain largely uncharacterized. To understand the molecular mechanism(s) by which β-sitosterol exerts its many beneficial health effects, we performed affinity chromatography using biotinylated β-sitosterol to identify its protein targets.

We reasoned that the health promoting effects of β-sitosterol not observed with cholesterol originate from the existence of unshared protein targets. β-Sitosterol differs from cholesterol solely by the presence of an ethyl group at the C-24 position, which we hypothesized would be an important moiety for the binding of β-sitosterol specific proteins. Therefore, we prepared affinity reagents for both compounds by attaching a biotin group to each through a polyethylene glycol linker (Fig. 1). The C-3 position was chosen as the attaching point because it is furthest away from the C-24 position and thus least likely to interfere with proteins that selectively bind β-sitosterol.

We performed affinity chromatography initially with lipopolysaccharide (LPS)-treated Raw264.7 macrophage cell lysates because many of the anti-inflammatory properties of β-sitosterol may arise from its effects on macrophages.[21,22] The lysates were first incubated with the biotinylated compounds or biotin alone as a control at various concentrations for 2 h, followed by overnight incubation of all with streptavidin agarose resin. SDS-PAGE and silver staining analysis revealed two bands that bound specifically to β-sitosterol, a 75-kDa band at 200 nM (Fig. 2A) and a 120-kDa band at 600 nM (Fig. 2B). MALDI mass spectrometry analysis of the bands identified them as 17-beta-hydroxysteroid dehydrogenase 4 (17β-HSD4) and extended synaptotagmin 1 (E-Syt1).

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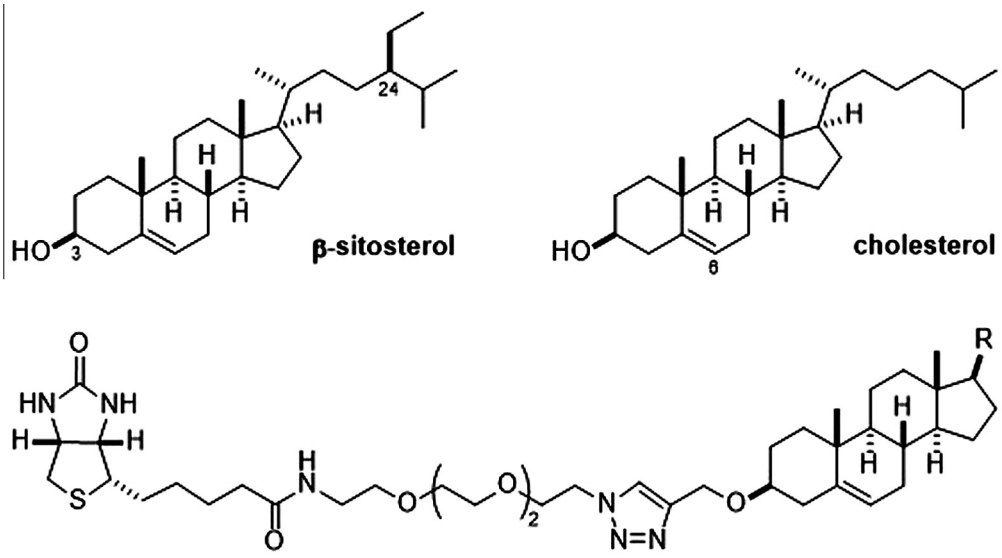

Figure 1. The structures of β-sitosterol, cholesterol, and the affinity reagents used herein.

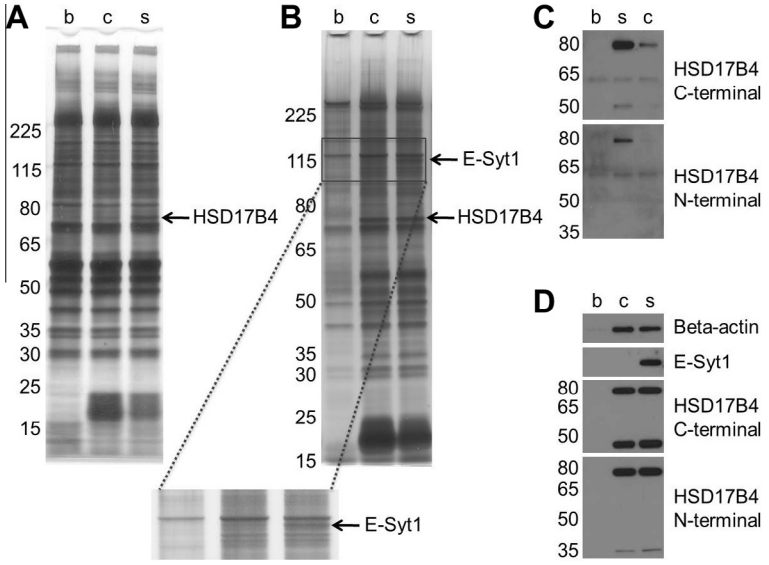

Figure 2. β-Sitosterol binds to 17β-HSD4 and E-Syt1 in lysates from LPS-treated Raw264.7 mouse macrophages. Affinity chromatography was performed using several concentrations of biotin (b), biotinylated cholesterol (c), and biotinylated β-sitosterol (s). Silver-staining and mass spectrometry analysis discovered two β-sitosterol specific binders: (A) 17β-HSD4 at 200 nM and (B) E-Syt1 at 600 nM. Immunoblotting analysis of the affinity chromatography samples validated the binding of (C) 17β-HSD4 at 200 nM and (D) E-Syt1 at 600 nM.

To validate these proteins we performed immunoblotting using separate aliquots of the affinity chromatography samples. As shown in Figure 2C, western blotting with two different 17β-HSD4 antibodies confirms that it is bound much more strongly by β-sitosterol than cholesterol. While the full length 17β-HSD4 polypeptide is 79 kDa, a portion of the cellular pool of 17β-HSD4 is proteolytically cleaved into two polypeptides, a 34-kDa N-terminal fragment and a 45-kDa C-terminal fragment.[23] These two polypeptides are stable within the cell and are thought to retain their enzymatic functions, either alone or as homodimers or heterodimers.[24–26] Although both fragments are present in the macrophage lysates, only the C-terminal fragment was bound by β-sitosterol (Fig. 2C). Interestingly, the C-terminal fragment contains a sterol carrier protein type 2 (SCP-2) domain, which may be the site of β-sitosterol and cholesterol binding.[27] Immunoblotting of the 600 nM affinity chromatography samples likewise validated that E-Syt1 bound specifically to β-sitosterol (Fig. 2D). At this concentration, 17β-HSD4 appears to be bound equally well by β-sitosterol and cholesterol, suggesting that the ethyl group at C-24 in β-sitosterol increases its affinity to 17β-HSD4 but is not necessary for binding.

Next, we extended our affinity chromatography studies to two prostate cancer cell lines, PC-3 and DU-145, because β-sitosterol has been reported to inhibit the growth, migration, and invasion of prostate cancer cells and is used to treat enlarged prostate.[7,15,28] As shown in Figure 3, E-Syt1 bound specifically to biotinylated β-sitosterol at 600 nM, just as in macrophage lysates. In contrast, 17β-HSD4 bound more strongly to biotinylated cholesterol than biotinylated β-sitosterol in both prostate cancer cell lines (Fig. 3), which is opposite to what we observed in macrophage lysates. β-Sitosterol has been reported to inhibit 5α-reductase at micromolar concentrations, and this inhibition has been hypothesized to be