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Phospholipidosis and down-regulation of the PI3-K/PDK-1/Akt signalling pathway are vitamin E inhibitable events associated with 7-ketocholesterol-induced apoptosis

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Abstract

Among the oxysterols accumulating in atherosclerotic plaque, 7-ketocholesterol (7KC) is a potent apoptotic inducer, which favours myelin figure formation and polar lipid accumulation. This investigation performed on U937 cells consisted in characterizing the myelin figure formation process; determining the effects of 7KC on the PI3-K/PDK-1/Akt signalling pathway; evaluating the activities of vitamin E (Vit-E) (α-tocopherol) on the formation of myelin figures and the PI3-K/PDK-1/Akt signalling pathway and assessing the effects of PI3-K inhibitors (LY-294002, 3-methyladenine) on the activity of Vit-E on cell death and polar lipid accumulation. The ultrastructural and biochemical characteristics of myelin figures (multilamellar cytoplasmic inclusions rich in phospholipids and 7KC present in acidic vesicles and the reversibility of these alterations) support the hypothesis that 7KC is an inducer of phospholipidosis. This oxysterol also induces important changes in lipid content and/or organization of the cytoplasmic membrane demonstrated with merocyanine 540 and fluorescence anisotropy, a loss of PI3-K activity and dephosphorylation of PDK-1 and Akt. It is noteworthy that Vit-E was able to counteract phospholipidosis and certain apoptotic associated events (caspase activation, lysosomal degradation) to restore PI3-K activity and to prevent PDK-1 and Akt dephosphorylation. When Vit-E was associated with LY-294002 or 3-methyladenine, impairment of 7KC-induced apoptosis was inhibited, and accumulation of polar lipids was less counteracted. Thus, 7KC-induced apoptosis is a PI3-K-dependent event, and Vit-E up- and down-regulates PI3-K activity and phospholipidosis, respectively.

Keywords: Akt; PI3-K; 7-ketocholesterol; Phospholipidosis; Vitamin E

1. Introduction

Oxysterols are 27 carbon derivatives of cholesterol containing additional oxygen atoms on the steroid's nucleus or on the side chain [1]. They are common components of oxidized lipoproteins (Ox-LDL), which play key roles at different stages of the atherosclerotic process [2] and comprise a large family of molecules resulting either from the auto-oxidation of cholesterol in air or from the enzyme-catalyzed transformation of cholesterol in various cell species [3]. Until now, the role played by oxysterols in the development of atherosclerotic lesions has been widely suspected for the following reasons: numerous studies

Abbreviations: AO, acridine orange; DMSO, dimethylsulfoxide; DPH, 1,6-diphenyl-1,3,5-hexatriene; FLICA, fluorochrome-labelled inhibitor of caspases; 7KC, 7-Ketocholesterol; MC540, merocyanine 540; 3MA, 3-methyladenine; MDC, monodansylcadaverine; NR, Nile Red; PDK-1, 3′Phosphoinositide-regulated kinase-1; PI3-K, phosphoinositide 3-kinase; PBS, phosphate-buffered saline; PI, propidium iodide; PP2A, protein phosphatase 2A; TMSE, Trimethylsilylethanol; TPBS, 0.1% Tween 20, phosphate buffered saline; Vit-E, vitamin E.

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determined increased oxysterol levels in the plasma of atherosclerotic patients and in atheromatous plaque [mainly 27-hydroxycholesterol, 7-ketocholesterol (7KC) and 7β-hydroxycholesterol] [4], and several investigations clearly established that some of these compounds (7β-hydroperoxycholesterol, 7β-hydroxycholesterol and 7KC) mimic the cytotoxic effects of Ox-LDL on the cells of the vascular wall [2]. Moreover, some oxysterols, especially 7KC and 7β-hydroxycholesterol, which are also present at important levels in some processed foods, have potent cytotoxic, pro-oxidative and/or proinflammatory properties [5,6], which are hallmarks of the pathophysiological mechanisms involved in the atherosclerotic process [2,3,7].

Of the different oxysterols capable of promoting atherogenesis, 7KC is one of the most abundant in plasma and arterial lesions of atherosclerotic patients [3]. It has been clearly established, particularly on human promonocytic U937 cells, that this oxysterol induces a complex mode of cell death with some characteristics of apoptosis: externalization of phosphatidylserine; loss of transmembrane mitochondrial potential ($\Delta\Psi$); mitochondrial release of cytochrome *c*; endonuclease G and apoptosis-inducing factor; cleavage of Bid; activation of caspases 2, 3, 7, 8 and 9; degradation of poly(ADP-ribose)polymerase and inhibitor caspase-activated deoxyribonuclease, internucleosomal DNA degradation and condensation/fragmentation or swelling of the nuclei, which are associated with the formation of multilamellar cytoplasmic structures [5,6,8]. These multilamellar cytoplasmic structures, isolated by subcellular fractionation after staining with monodansylcadaverine (MDC), have been partially characterized: they accumulate 7KC and also contain high amounts of phosphatidylcholine and sphingomyelin [9]. It is noteworthy that ultrastructurally similar MDC-positive multilamellar bodies, considered autophagic vacuoles, were observed in Mv1Lu mink lung type II alveolar cells transfected with β1-6-*N*-acetylglucosaminyl transferase V [10]. Based on ultrastructural and biochemical criteria, it was assumed that 7KC-induced cell death might also be associated with an autophagic process [11,12]. Moreover, since a close relation may exist between autophagy and apoptosis [13], and since the phosphoinositide 3-kinase (PI3-K)/Akt pathway is an important second messenger system involved in both autophagy [14] and apoptosis [15], it was important to determine the role played by the PI3-K/Akt signalling pathway in 7KC-induced cell death. Akt, which was initially identified as the mammalian homologue of the viral oncogene v-akt, is also called "protein kinase B" (PKB) [16]. A probable inactivation of the PI3-K/Akt signalling pathway in 7KC-induced cell death was suggested by a previous investigation demonstrating an activation of the proapoptotic protein BAD (Bcl-xl/Bcl-2 associated death promotor), which is present in its dephosphorylated form in 7KC-treated cells [17]. Indeed, when Akt is activated and presents in its phosphorylated form, via the PI3-K kinase signalling pathway through the 3-phosphoinositide-dependent protein kinase-1 (PDK-1), it maintains BAD in its inactive form by phosphorylation on serine 99 [15].

Based on these different considerations, the aim of the present study conducted on untreated and 7KC-treated human promonocytic U937 cells and, in part, on rat A7R5 aortic smooth muscle cells, was: (1) to characterize the cellular process associated with the formation of multilamellar structures (also called myelin figures) observed with 7KC treatment as well as with other cytotoxic oxysterol treatments [5,8,11] and to rely the formation of these myelin figures with cell death; (2) to determine the effects of 7KC on the PI3-K/PDK-1/Akt signalling pathway; (3) to evaluate the effects of vitamin E (Vit-E; α-tocopherol) on the formation of myelin figures and on the PI3-K/PDK-1/Akt signalling pathway, since we previously described an impairment of 7KC-induced apoptosis by Vit-E [18]; and (4) to determine the effects on various PI3-K inhibitors (LY-294002, 3-methyladenine) on the activity of Vit-E. The role played by the protein kinase PI3-K was investigated since Akt/PKB regulates B-cell lymphoma (BCL) family members during oxysterol-induced apoptosis [19].

We report that 7KC-induced myelin figures are acidic phospholipid-rich vesicles, also accumulating 7KC and cholesterol. Thus, it was demonstrated that 7KC is a potent inducer of phospholipidosis [20,21], which precedes early signs of cell death such as the loss of transmembrane mitochondrial potential and morphological nuclear changes. In addition, we show that 7KC-induced cell death and phospholipidosis are counteracted by Vit-E, which is also capable of restoring the loss of PI3-K activity and the dephosphorylation of PDK-1 and Akt triggered by 7KC. However, the impairment of 7KC-induced apoptosis by Vit-E was inhibited by LY-294002 and 3-methyladenine, and the decrease in polar lipid accumulation was almost abolished when Vit-E was associated with LY-294002 and 3-methyladenine.

2. Materials and methods

2.1. Cells and treatments

Human promonocytic leukaemia cells (U937) obtained from the American Type Culture Collection (Manassas, VA, USA) were used. U937 cells were grown in RPMI 1640 with GlutaMAX I (Gibco, Eragny, France) and antibiotics (Invitrogen, Cergy-Pontoise, France) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco); they were seeded at 500,000/ml culture medium and passaged twice a week.

The 7KC was provided by Sigma (L'Isle d'Abeau Chesnes, France), and its purity was determined to be 100% by gaseous phase chromatography coupled with mass spectrometry. For all experiments, a stock solution of 7KC was prepared at a concentration of 800 μg/ml, as previously described [5]. In all experiments conducted on U937 cells, 7KC was added to the culture medium containing 10% heat-