Effects of 7-ketocholesterol on the activity of endothelial poly(ADP-ribose) polymerase and on endothelium-dependent relaxant function

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Abstract. Oxidative and nitrosative stress play an important role in the development of endothelial vascular dysfunction during early atherosclerosis. Oxidative stress activates the nuclear enzyme poly(ADP-ribose) polymerase (PARP) in endothelial cells. In patients with atherosclerosis the level of oxidized LDL in the plasma is elevated. In oxidized LDL various oxysterols have been identified, such as 7-ketocholesterol (7K). 7K has been shown to induce PARP activation in microglial cells. The aim of the current study was to clarify the effects of 7K on the activity of endothelial PARP and on the endothelium-dependent relaxant function of blood vessels. We treated human umbilical vein endothelial (HUVEC) cells with 2-16 μg/ml 7K as well as vascular rings harvested from BALB/c mouse thoracic aorta with 90 μg/ml 7K for 2 h. A group of mice was treated with 7K subcutaneously for 1 week (10 mg/kg/day). We also conducted *in vitro* and *in vivo* experiments using pretreatment with buthionine sulphoximine (BSO), a glutathione-lowering agent. The activity of PARP was calculated by measurement of tritiated NAD incorporation. The activity of PARP increased significantly in 7K-treated HUVEC cells. After BSO pretreatment, this increase was higher. Isolated vascular rings demonstrated no change in endothelium-dependent relaxant function after 2 h of incubation with 7K, even after BSO pretreatment. *In vivo* treatment with 7K for 1 week had no effect on the relaxant function. Our experimental results suggest that although 7-ketocholesterol can activate PARP enzyme in endothelial cells, it is not sufficient on its own to cause impairment in the endothelium-dependent vascular reactivity.

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Introduction

Oxidative and nitrosative stress play an important role in the pathogenesis of atherosclerosis (1,2). Elevated levels of oxidative DNA damage have been shown in atherosclerotic plaques (3). Oxidative DNA damage activates the PARP enzyme. PARP is an abundant nuclear enzyme, whose over-activation promotes cell death (4). PARP also plays a role in neointima formation (5).

Low density lipoproteins are the major vehicles transporting cholesterols in plasma. In patients with atherosclerosis the LDL levels are usually high and the oxidation of these particles is considered to be a major factor in their atherogenicity (6). One of the main oxysterols in oxidized LDL (oxLDL) is 7-ketocholesterol (7). 7-Ketocholesterol (7K) was shown to inhibit arterial endothelium-dependent relaxation in rabbit aortas (8) and was reported to activate PARP in microglial cells (9), although the latter occurs at very high 7K concentrations (10).

PARP activation may play a role in the pathogenesis of atherosclerosis, as inhibition improves the endothelial function of apoE-deficient mice (11). In the context of examining the potential role of PARP and 7K in the early stage of atherosclerosis, here we investigated whether 7K induces PARP-activation in endothelial cells, and we examined its role in the development of endothelial dysfunction.

Materials and methods

Measurements of PARP activation in HUVEC cells. Human umbilical vein endothelial (HUVEC) cells were plated at a density of 250,000 cells per well in a 12-well plate and grown in M199 media supplemented with 10% fetal calf serum and 0.03 mg/ml endothelial cell growth supplement. After 24 h, the media was replaced and the cells were treated with increasing concentrations of 7K (2, 4, 8, 16 μg/ml) for 24 h with or without L-buthionine-(S,R)-sulphoximine (BSO, 1 mM). In subsequent experiments HUVEC cells were treated with H_2O_2 (positive control) or with 20 μg/ml 7K with or without pretreatment with the PARP inhibitor, PJ-34 (3 μM, 1 h) or the nitric oxide synthase (NOS) inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 3 mM, 1 h). For

the measurement of cellular PARP activity (12,13), the media was removed and replaced with 0.5 ml N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (pH 7.5) containing 0.01% digitonin and 3H-NAD (0.5 μCi/ml), and the cells were incubated for 20 min at 37˚C. The cells were then scraped from the wells and placed in Eppendorf tubes containing 200 μl ice-cold 50% trichloroacetic acid (TCA) (w/v). The tubes were then placed at 4˚C. After 4 h, the tubes were centrifuged at 1800 x g for 10 min and the supernatant removed. The pellet was washed twice with 500 μl ice-cold 5% TCA. The pellet was solubilized in 250 μl NaOH (0.1 M) containing 2% sodium dodecyl sulfate overnight at 37˚C. The PARP activity was then determined by measuring the radioactivity incorporated using a Wallac scintillation counter (Wallac, Turku, Finland). The solubilized protein (250 μl) was mixed with 5 ml scintillant (ScintiSafe Plus, Fisher, Pittsburgh, PA, USA) before being counted for 10 min. Results are expressed as a percentage of the PARP activity observed in untreated cells. Cell viability was measured by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay and was unaffected by the pharmacological inhibitors used.

Measurement of isometric force in aortic rings of mice. BALB/c mice were treated for 1 week with 7-K or vehicle applied subcutaneously at a daily dose of 10 mg/ kg. The method to determine endothelium-dependent vascular relaxation in thoracic aortic rings from mice was described previously (14). Briefly, the thoracic aorta was cleared from periadventitial fat and cut into 3 mm-width rings using an operation microscope, mounted in organ baths filled with warmed (37˚C) and oxygenated (95% O_2, 5% CO_2) Krebs' solution ($CaCl_2$, 1.5 mM; $MgSO_4$, 1.2 mM; NaCl, 118 mM; $NaHCO_3$, 14.8 mM; KCl, 4.6 mM; NaH_2PO_4, 1.2 mM; glucose, 11.1 mM). Isometric tension was measured with isometric force transducers (HBM, Q11), which were connected to a transducer-amplifier (HBM, MGA II) and recorded on paper (Kipp Zonen, BD300). A tension of 1 g was applied, and the rings were equilibrated for 60 min. Fresh Krebs was provided at 20-min intervals. After equilibration the contractile response of arterial rings to a depolarizing solution of a modified Krebs solution enriched in K^+ (124 mM) was initially tested to evaluate their functional integrity. For the measurement of endothelial functionality, the rings were precontracted with phenylephrine (10^{-6} M) and then dose-response curves to acetylcholine (10^{-8} M-10^{-5} M) were constructed. Experiments were conducted on 20 rings in each experimental group.

In a separate set of experiments vascular rings were incubated with 7K (90 μg/ml) or vehicle for 2 h, followed by the determination of endothelium-dependent vascular function.

In both sets of experiments subgroups were pretreated with 1g/kg BSO/24 h for the inhibition of γ-glutamylcysteine synthetase, thus lowering the antioxidant capacity (15).

The investigation was performed with the approval of the Institutional Animal Care and Use Committee.

Immunohistochemical analysis of PAR. Tissues were fixed in 4% buffered formalin and paraffin sections were prepared.

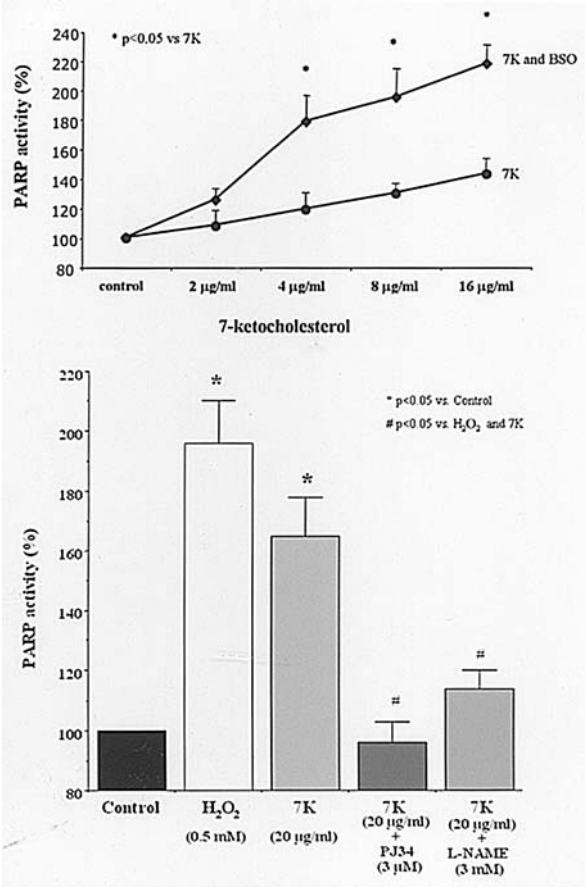

Figure 1. 7-Ketocholesterol treatment activates PARP in HUVEC cells in a dose-dependent manner. Further analysis of this effect shows that BSO pretreatment augments this activation suggesting an oxidant effect of 7K (upper panel). The degree of PARP activation by 7K is comparable to the degree elicited by H_2O_2. 7K-induced PARP activation is blocked by PARP inhibitor PJ34 and the non-isoform-selective NOS inhibitor, L-NAME (lower panel). Data represent mean ± SD.

Sections were treated with 0.6% H_2O_2 in methanol to quench endogenous peroxidase activity and microwaved in 0.2 M citrate buffer (pH 3.0) to retrieve antigenic epitopes. After blocking in 1.5% normal goat serum, a polyclonal anti-PAR antibody (Calbiochem, San Diego, CA, USA) was applied at a 1:100 dilution. A biotinylated secondary antibody and the ABC method were used to visualize PAR polymers with 3, 3'-diaminobenzidine-tetrahydrochloride (DAB) and H_2O_2 as substrate (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with Gill's hematoxyline (Accustain, Sigma Diagnostics, St. Louis, MO, USA).

Statistical analysis. Results are reported as mean ± SD. Statistical significance between measurements was determined by ANOVA followed by a *post hoc* Dunnett test. Probability values of $p<0.05$ were considered significant.

Results

7-Ketocholesterol activated PARP in a dose-dependent manner. This activation was higher with the pretreatment of

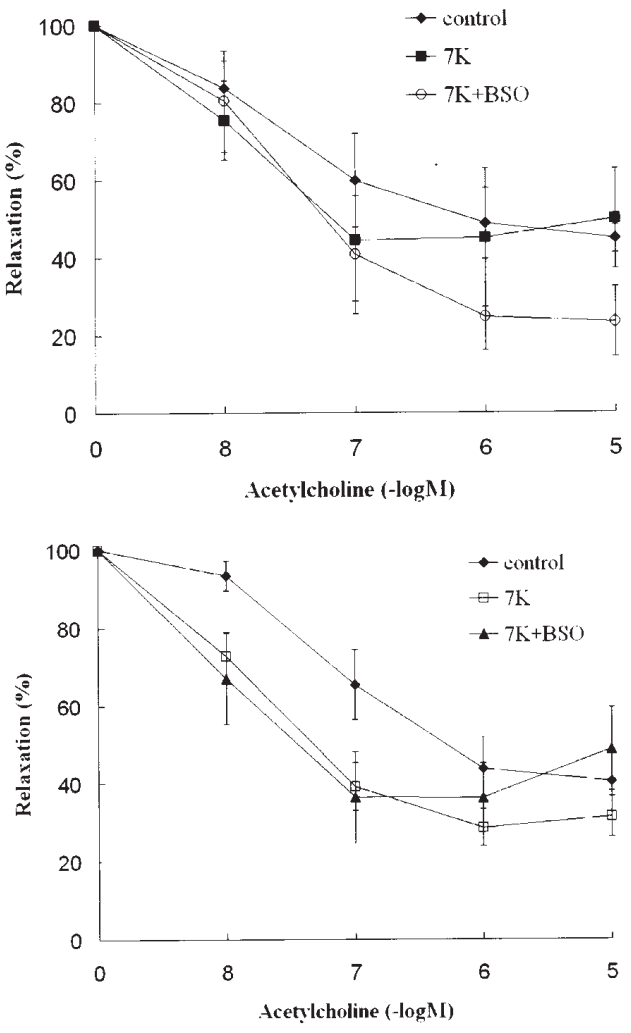

Figure 2. The effect of 7-ketocholesterol treatment on endothelium-dependent vasodilation. Incubation with 90 μg/ml for 2 h causes no significant dysfunction compared to control (upper panel). Pretreatment of mice with 10 mg/kg/day 7K causes no difference compared to vehicle-treated animals (lower panel). Data represent mean ± SD.

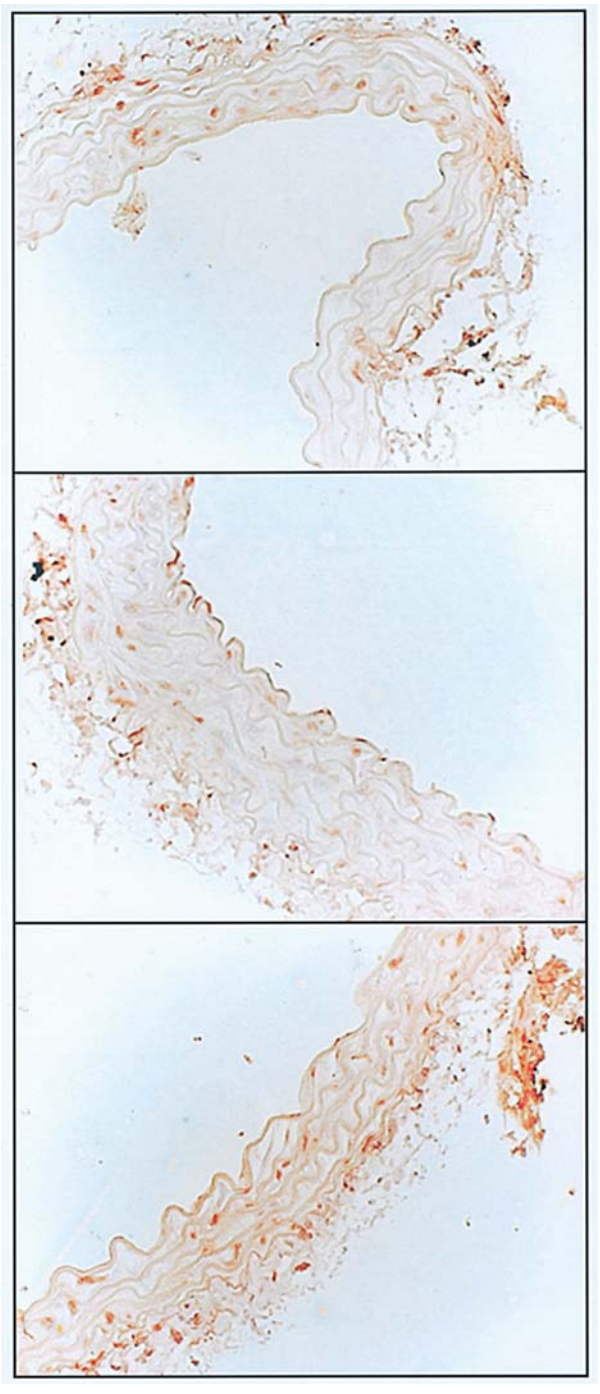

Figure 3. The PARP activity of endothelial cells in the vessel wall shows no significant difference between the groups studied (from top: 7K incubation, 7K pretreatment, control). Anti-PAR staining (1:100), hematoxylin background.

BSO (Fig. 1, upper panel). Further analysis of this activation showed that 7K had nearly the same potential in activating PARP as H_2O_2, and this activation was able to be blocked by the PARP inhibitor PJ34 and by the non-selective NOS-inhibitor L-NAME (Fig. 1, lower panel).

The experiments conducted on the vascular rings showed no difference in Ach-induced relaxations, neither after incubation of rings with 7K, nor after subcutaneous treatment of mice with 7K, even in the presence of BSO pretreatment (Fig. 2).

The PARP activity of endothelial cells in the vessel wall showed no significant difference between the groups studied (Fig. 3.).

Discussion

Several studies have examined the role of oxysterols found in oxLDL in the early pathogenesis of atherosclerosis. The excess concentration of oxysterols was shown to be cytotoxic to endothelial cells inducing apoptosis (7,16,17). Other investigations showed apoptosis of vascular smooth muscle cells after treatment with cholesterol-oxides (18). Mougenot *et al* measured arterial vasomotor responses related to oxidized LDL and indicated that the major cholesterols contributing to the atherogenic effect were 7-ketocholesterol and 7ß-hydroxicholesterol (19). 7K was shown to inhibit arterial endothelial-dependent relaxation in the rabbit aorta (8). Incubating BV-2 microglial cells with 7K caused PARP-activation (9). However the possible relation between 7K and PARP in the context of endothelial dysfunction has not yet been investigated.

Poly(ADP-ribose) polymerase-1 (PARP), a monomeric nuclear enzyme present in eukaryotes, is the major isoform of an expanding family of poly(ADP-ribosyl)ating enzymes. The main isoform of the family, PARP-1, functions primarily as a DNA damage sensor. Upon binding to damaged DNA mainly through the second zinc finger domain, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins. The biological role of PARP-1 includes the regulation of DNA repair and maintenance of genomic integrity (20). PARP has been implicated in a variety of pathophysiological processes. PARP overactivation, in response to severe oxidant-induced DNA damage, has been shown to promote cell dysfunction culminating in cell necrosis (21).

Here we demonstrated for the first time that 7-keto-cholesterol (the main oxysterol found in oxLDL) is capable of activating PARP in endothelial cells. This activation appears to be related to NOS activity because L-NAME attenuated the effect of 7K. The phenomenon of PARP activation also appears to be dependent on intracellular oxidative stress, as pretreatment with BSO increased the degree of PARP activation. One likely scenario is that the constitutively produced NO in endothelial cell combines with the superoxide, which, in turn, results in the formation of peroxynitrite, which is a known endogenous trigger of DNA single-strand breakage and PARP activation. In this context, endothelial NO/peroxynitrite production may result in endothelial dysfunction, which subsequently may damage the endothelial function, and may lead to impaired endothelial NO production later on. Such impaired NO production has previously been demonstrated after 7K treatment (8,22,23).

Direct incubation of the rings with 7K or subcutaneous treatment with 7K did not inhibit endothelial-dependent relaxation in our experimental settings and the endothelial cells in the vessel walls showed no PARP-activation, which could have been due to the effect of the rapid hepatic metabolism of 7K (24,25).

To test the effect of 7K in a state where the vasculature may become more sensitive to oxidant stress, we utilized BSO pretreatment in mice. In previous studies it was shown that in vascular rings taken from rats which had been pretreated with BSO to deplete endogenous glutathione, there was a significantly more pronounced suppression of the contractility and a significantly more pronounced (near-complete) inhibition of the endothelium-dependent relaxations after peroxynitrite exposure (15). Here we found no evidence that *in vivo* 7K leads to apoptosis and endothelial dysfunction, even after the depletion of glutathione.

Based on the current findings, 7K is certainly capable of PARP activation in endothelial cells, but it is unlikely that 7K, on its own, leads to endothelial dysfunction, although it is possible that local accumulation of 7K in plaques may lead to higher, more cytotoxic concentrations. It is probable that 7K acts in synergy with other pro-atherosclerotic molecules in the early stage of atherosclerosis.

Data in the literature demonstrate that 7K can activate a variety of intracellular pathways such as PKC activation (8), activation of ubiquitin complexes (26), calcium-dependent activation of several pro-apoptotic pathways and also the MEK→ERK survival pathway (27), upregulation of the NAD(P)H oxidase homologue Nox-4 (28) or modulation of the Ca^{++} signal and inhibition of eNOS (22). The wide variety of possible effects indicates that 7K has numerous targets in the cells of the vascular wall, one of which could be the PARP-pathway, and the combined effects of the activation of these pathways may lead to vascular cell damage and cell death.

Collectively, our results suggest that although the main oxysterol component of oxLDL particles, 7-ketocholesterol, is capable of activating PARP in endothelial cells, it cannot induce endothelial dysfunction alone. It is conceivable that 7-ketocholesterols and other oxysterols found in oxLDL together may be responsible for the development of endothelial dysfunction induced by these lipoproteins.

Acknowledgements

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