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Early Involvement of ROS Overproduction in Apoptosis Induced by 7-Ketocholesterol

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ABSTRACT

Cholesterol oxidation products are increasingly considered as much more bioactive than the parent compound in the mutifactor and multistep process that characterizes atherosclerosis. In particular, 7-ketocholesterol has been reported to induce oxidative stress as well as a marked pro-apoptotic effect in vascular cells including macrophages. With the aim to investigate a possible pathogenic correlation between the two events, cultivated murine macrophages were challenged with a concentration of 7-ketocholesterol actually detectable in human vasculature. Conclusive proof was obtained of a primary role of NADPH-oxidase in the overproduction of reactive oxygen species within cells treated with the oxysterol. In addition, such oxidative burst occurred very early after cell intoxication and it was definitely demonstrated as able to lead cells to apoptotic death. In fact, two metabolic inhibitors of NADPH-oxidase and the antioxidant epicatechin very well counteracted 7-ketocholesterol-induced apoptosis by preventing the oxysterol pro-oxidant action. *Antioxid. Redox Signal.* 8, 375–380.

INTRODUCTION

ALTHOUGH HYPERCHOLESTEROLEMIA is constantly associated with the vascular alterations of atherosclerosis (11, 13, 18), as yet undefined are the mechanisms by which cholesterol may contribute to the expression or better to the progression of such major disease process. Over the last few years it became clear that the driving force of atherosclerosis progression is an inflammatory process triggered and sustained by oxidized low density lipoprotein (LDL) (16, 19). Cholesterol, in free and esterified form, represents about half the weight of a LDL micelle. Thus, LDL lipid oxidation certainly involves the sterol in a primary way, with the consequent formation of cholesterol oxidation products, 27-carbon compounds termed oxysterols. Indeed, a number of oxysterols have consistently been detected both in plasma of humans, in particular hypercholesterolemic ones, as well as in atheromas (i.e., the atherosclerotic lesions) (1, 4, 7). Of the various biochemical effects shown by certain oxysterols among those recovered in human plasma and fibrotic plaques, the potential pro-apoptotic action of some of them is interesting. A large bulk of literature is now available on the apoptotic effect exerted by oxysterols on the different cell types present in the vasculature (for a review, see Ref. 9). Of note, nearly all these studies agree on at least one point: the type of irreversible damage provoked to endothelial cells, smooth muscle cells and cells of the macrophage lineage, which is apoptosis and not necrosis. The constant finding of programmed cell death in the arterial wall tracts affected by lipid accumulation and sclerosis is not in conflict at all with the now general opinion that inflammation is a key event in both formation and evolution of the atherosclerotic lesion. In fact, contrary to the previous general belief, an increasing bulk of evidence is demonstrating that apoptosis may trigger inflammatory stimuli exactly as necrosis (5, 8).

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Another consistent feature of the atherosclerotic disease process is a degree of redox imbalance towards oxidation within the lesion. Accumulating LDLs are perhaps already partly oxidized, and macrophages become activated and undergo oxidative bursts to generate excessive amounts of reactive oxygen species (ROS) that promote further lipid oxidation. In addition, it seems that at least certain oxysterols are able to amplify such an oxidative stress condition present in the atheroma by contributing to ROS formation through the upregulation of NADPH-oxidase activity (14, 15). We recently confirmed that 7-ketocholesterol (7K), one of the most toxic oxysterols of pathophysiological relevance, was able to induce ROS overproduction in macrophages (2). Here we report on conclusive demonstration that ROS overproduction by 7-ketocholesterol represents the initiating event in the apoptotic process induced by this sterol oxide.

MATERIALS AND METHODS

Cell culture and treatment

Murine macrophage J774A.1 cells were grown in RPMI-1640 medium (Sigma-Aldrich, Milano, Italy) supplemented with 20% fetal bovine serum (Life Technologies, Italia Srl, S. Giuliano Milanese, Italy), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine (Sigma-Aldrich) at 37°C in humidified 95% air/5% CO_2. The cells were dispensed at 1 million/ml and made quiescent through overnight incubation in serum-free medium. Cells were then placed in RPMI-1640 medium with 2% fetal bovine serum and treated with 7-ketocholesterol (Steraloids, Newport, RI, USA) at final concentration of 20 μ*M* and incubation times as indicated in the figure legends. Other cells were pretreated with 1 μ*M* diphenylene iodonium (DPI) or cotreated with 50 μ*M* 4-aminoethyl-benzenesulfonylfluoride (AEBSF), both NADPH oxidase inhibitors, or pretreated with 5–10 μ*M* antioxidant epicatechin (Sigma-Aldrich).

The solvent used for diluting the oxysterol and epicatechin was ethanol, while the solvent used for diluting DPI was dimethyl sulfoxide (DMSO). Untreated cells but incubated with the same amount of alcohol and/or DMSO were used as solvent controls (see figure legends).

Cell viability was routinely checked by the Trypan blue exclusion method. In all experimental groups but one, cell viability ranged between 80% and 90%. Only cells pretreated with DPI showed a slight decrease of viability.

Measurement of intracellular production of reactive oxygen species by confocal laser scanning fluorescence microscopy

To measure the oxysterol-induced intracellular ROS, cells were grown in thick-glass base dishes. Cells were treated with 7-ketocholesterol plus or minus DPI, AEBSF, or epicatechin, and incubated in the dark with 10 μ*M* 2′,7′-dichlorofluorescin-diacetate (DCFH-DA)/0.1 *M* phosphate buffered saline (PBS) solution for 30 min at 37°C. The DCFH-DA (Sigma-Aldrich) was cleaved into the cells to 2′,7′-dichlorofluorescin (DCFH) and oxidized by ROS to fluorescent 2′,7′-dichlorofluorescein (DCF) which was monitored directly by confocal laser microscopy.

ROS production were analyzed by a laser scanning confocal microscope Zeiss LSM 510 (Carl Zeiss SpA, Arese, Milano, Italy) equipped with a Zeiss inverted microscope with a "plan neofluar" lens: 20×/0.5 and 40×/0.75 for DCF fluorescence evaluation. Exciting light intensity, black level, and photomultiplier gain were adjusted on control specimens; settings were the same for scanning experimental samples. The instrument was set to 488 nm exciting laser band, with a 515 nm long pass emission filter. The DCF fluorescent images were elaborated as arbitrary units/pixel using a LSCM 510 Image Examiner Program from Zeiss; the lens used was 20×/0.5 (0.849 x 0.849 mm^2 image dimension).

Nuclear morphology by Hoechst staining

Changes in nuclear morphology were determined after harvesting cells by centrifugation at 80 *g* for 5 min, washing in 0.1 *M* PBS, pH 7.4, resuspension to 0.2 × 10^6 cells/ml and application to glass slides (8 × 10^4 cells/slide) by cytocentrifugation at 20 *g* for 5 min (Cytospin cytocentrifuge, Shandon Inc., Pittsburgh, PA, USA). After fixing with 95% ethanol for 5 min at room temperature, the cells were washed with 0.1 *M* PBS and incubated for 30 min at room temperature in 0.8 μ*M* Hoechst-33342/0.1 *M* PBS solution. All slides were then washed three times and sealed with glycerol. Nuclear morphology was examined using a fluorescent microscope LEICA PMIRE-2 with an ultraviolet filter and a 40× oil-immersion lens (Leica Microsystem Wetzlar GmbH, Wetzlar, Germany).

Evaluation of caspase-3 activity

At the end of the incubation times, confluent cells (1 × 10^7/flask) were harvested by centrifugation at 80 *g* for 5 min, washed with cold 0.1 *M* PBS and resuspended in 600 μl ice cold lysis buffer [25 m*M* HEPES, 5 m*M* $MgCl_2$, 5 m*M* EDTA, 5 m*M* dithiothreitol (DTT), 2 m*M* phenylmethylsulfonyl fluoride (PMSF)]. After 15 min in ice, the suspension was frozen/thawed five times (liquid nitrogen/37°C), centrifuged at 10,000 *g* for 30 min at 4°C and the supernatant was collected.

The samples were diluted with distilled water to achieve a protein concentration of 0.5 mg/ml, and incubated for 100 min at 37°C with the fluorogenic substrate solution at a final concentration of 65 μ*M* [fluorogenic substrate was dissolved in 60 m*M* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 303 m*M* NaCl, 5 m*M* DTT, 5 m*M* EDTA, 5 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 30% sucrose (w:v)]. Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Vinci Biochem, Firenze, Italy), as caspase-3 substrate, was used. Caspase specific activities were evaluated as release of fluorescent AMC, using a spectrofluorometer (Kontron SFM 25, USA) set to 380 nm excitation and 510 nm emission and calibrated with a 1 m*M* AMC standard solution.

Proteins were evaluated with a Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, CA, USA), following the Bradford method (3).

Statistical analyses

The statistical significance of the differences between experimental groups was analyzed with the one-way ANOVA test associated with the Bonferroni's post test.

RESULTS

Early increase of ROS steady-state level in 7-ketocholesterol-treated macrophages

The incubation of murine J774A.1 macrophages with 7K at the final concentration of 20 μ*M*, induced a significant increase of intracellular ROS as early as 30 min after treatment, as shown by confocal microscopy analysis of DCF fluorescent reaction (Fig. 1). Fluorescence intensity in cells challenged with the sterol kept increasing with time, practically involving all treated cells within 3 h incubation. On the contrary, internal control, (i.e., macrophages incubated in the presence of the oxysterol solvent only), did consistently show fluorescence limited to few scattered cells all through the experimental observation (Fig. 1).

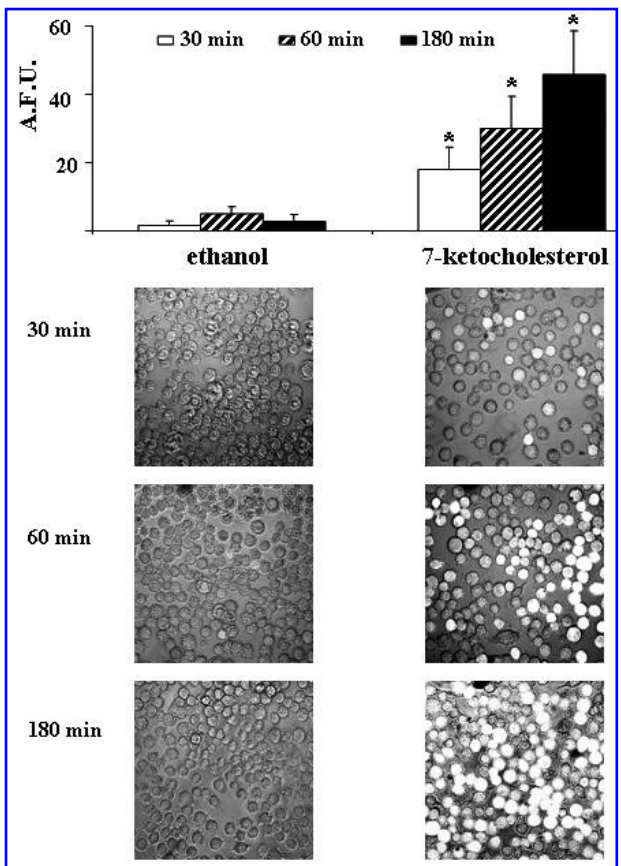

FIG. 1. Early increase of ROS steady-state level in 7K-treated macrophages. Cells were incubated for 30, 60, or 180 min with 20 μ*M* 7-ketocholesterol dissolved in 12.5 μ*M* ethanol or with 12.5 μ*M* ethanol alone. ROS production was visualized in macrophages as DCF fluorescence by laser scanning confocal microscopy (as described in Materials and Methods). Photographs are from one representative experiment ("plan neofluar" lens 40×/0.75). On the contrary, histograms report the fluorescence in arbitrary units/pixel (A.F.U.) and are mean values ± SD of three different experiments. *Significantly different vs. corresponding ethanol groups ($p < 0.001$).

Prevention of 7-ketocholesterol-dependent excessive ROS generation in macrophages by NADPH-oxidase metabolic inhibitors

In order to check the involvement of NADPH-oxidase in the observed increase of ROS steady-state levels in J774A.1 macrophages treated with 20 μ*M* 7K, pre- or co-incubation with two different selective inhibitors of NADPH-oxidase**,** 1 μ*M* DPI (10) or 50 μ*M* AEBSF (6) was performed. As shown in Figure 2 (upper panel), DPI was able to markedly but not

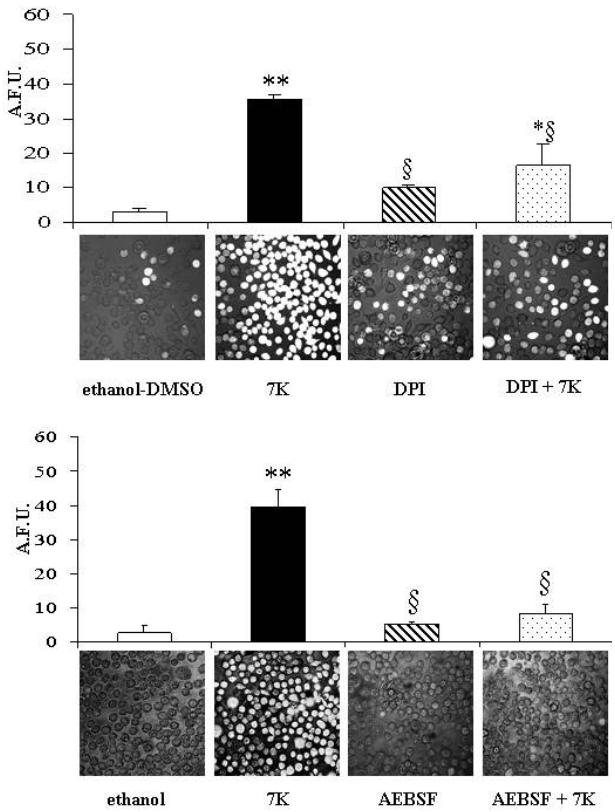

FIG. 2. Prevention of 7K-dependent excessive ROS generation in J774A.1 macrophages by NADPH-oxidase metabolic inhibitors. DCF fluorescence formation was visualized by laser scanning confocal after 180 min cell incubation with 20 μ*M* 7K, dissolved in 12.5 μ*M* ethanol, in the presence or in the absence of NADPH-oxidase inhibitors. DPI (1 μ*M*), dissolved in DMSO (70.4 μ*M*), was added to cell cultures for 30 min then removed before 7K treatment. AEBSF (50 μ*M*) was added to the cells together with 7K. The fluorescence intensity was quantified with an associated image analysis software as described in the previous figure legend. Histograms report the arbitrary units/pixel (A.F.U.) of fluorescence as means ± SD of three different experiments. Once again, photographs are from one representative experiment. *Significantly different vs. ethanol or ethanol-DMSO ($p < 0.01$); **significantly different vs. ethanol or ethanol-DMSO ($p < 0.001$); §significantly different vs. 7K ($p < 0.001$).

completely inhibit 7K-induced ROS overproduction. In this relation, even more pronounced was the protection against 7K afforded by AEBSF (Fig. 2, lower panel). In the same experiment, DPI appeared to exert *per se* a modest increase of fluorescence, but this finding was inconsistent and not statistically significant versus ethanol-DMS0 (Fig. 2, upper panel).

Prevention of 7-ketocholesterol-induced apoptotic effect in cultivated macrophages by NADPH-oxidase metabolic inhibitors

The already recognized pro-apoptotic action of 7K against vascular cells was reproduced in J774A.1 murine macrophages by the single addition of 20 μ*M* oxysterol and the process was checked morphologically in terms of Hoechst staining after 24 h treatment. As illustrated in Figure 3, challenge with 7K led to a significant increase of condensed nuclei in macrophages, as to control samples (Fig. 3A), semiquantitatively estimated as involving about 30% of the cells/microscopic field (Fig. 3B). When cells underwent 30 min pretreatment with 1 μ*M* DPI before 24 h incubation with 20 μ*M* 7K, only about 12% of the cells/microscopic field showed apoptotic changes (Fig. 3D). Macrophages pretreated with DPI then incubated 24 h in the absence of 7K exhibited just a few cells with condensed nuclei (Fig. 3C). Definitely consistent findings were obtained when 7K-treated J774.A.1 cells were simultaneously incubated in the presence of 50 μ*M* AEBSF (Fig. 4A–D).

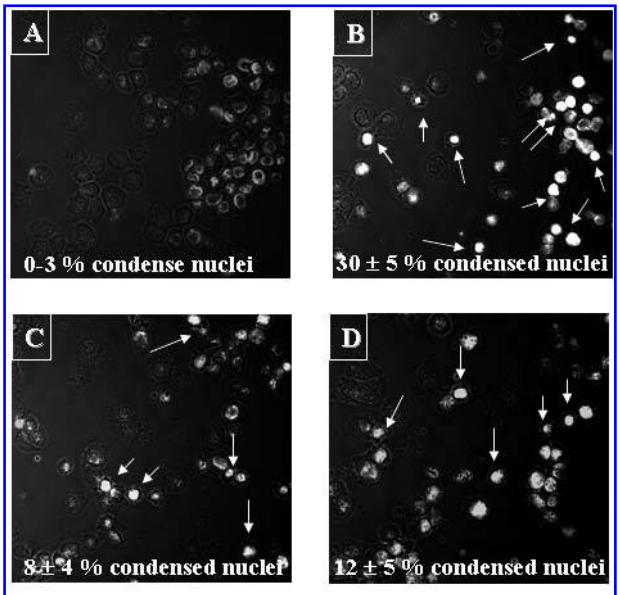

FIG. 3. Morphological evidence of 7K-induced apoptosis by Hoechst staining in J774A.1 macrophages; prevention by cell pretreatment with DPI. Cells were pretreated for 30 min with 1 μ*M* DPI, then, after change of the medium, incubated 24 h in the presence of 7K. *Arrows* indicate condensed nuclei; the fluorescence was detected using a LEICA PMIRE-2 microscope with an ultraviolet filter and a 40× oil immersion lens. (**A**) 70.4 μ*M* DMSO + 12.5 μ*M* ethanol; (**B**) 20 μ*M* 7K; (**C**) 1 μ*M* DPI; (**D**) 1 μ*M* DPI + 20 μ*M* 7K. The quantification of condensed nuclei was done counting cells in at least 10 fields per experimental group and was expressed as percentage of the number of cells/field ± SD.

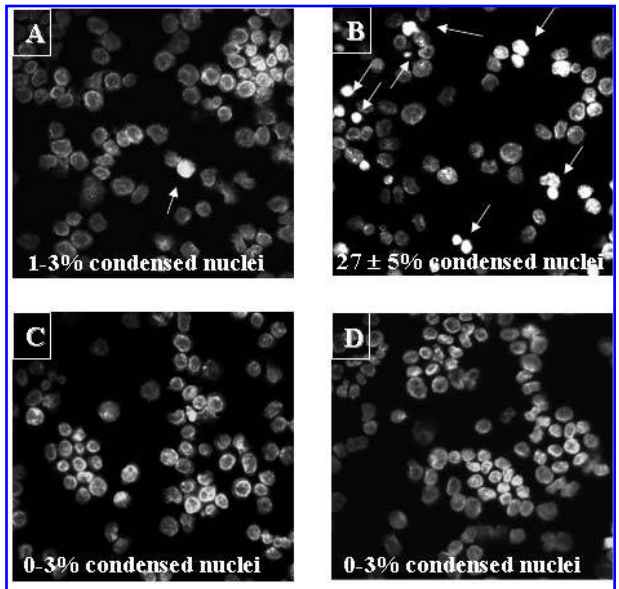

FIG. 4. Morphological evidence of 7K-induced apoptosis by Hoechst staining in J774A.1 macrophages; prevention by cell AEBSF treatment. Cells were treated for 24 hours with 20 μ*M* 7K, in ethanol, plus or minus 50 μ*M* AEBSF. *Arrows* indicate condensed nuclei; the fluorescence was detected using a LEICA PMIRE-2 microscope with an ultraviolet filter and a 40× oil immersion lens. (**A**) 12.5 μ*M* ethanol; (**B**) 20 μ*M* 7K; (**C**) 50 μ*M* AEBSF; (**D**) 20 μ*M* 7K + 50 μ*M* AEBSF. The quantification of condensed nuclei was done counting cells in at least 10 fields per experimental group and was expressed as percentage of the number of cells/field ± SD.

Prevention of 7-ketocholesterol-dependent increase of intracellular ROS in macrophages pretreated with epicatechin

Aiming to prove a cause-effect relationship between ROS overproduction and occurrence of apoptosis in macrophages treated with 7K, epicatechin, a widely used antioxidant flavonoid, was employed. Due to the described ability of this flavonoid to possibly inhibit NADPH-oxidase, different amounts of epicatechin were tested. The final concentration of 10 μ*M* was eventually adopted, since not inhibiting *per se* either the macrophage constitutive ROS production (Fig. 5) or cell viability (not shown). Under such experimental conditions, 7K once more confirmed to dramatically upregulate ROS level in treated cells after 3 h incubation, while 60 min pretreatment with epicatechin exerted indeed a quite prominent protection (Fig. 5).

Prevention of caspase-3 upregulation in 7-ketocholesterol-incubated macrophages by epicatechin

Pro-apoptotic effect of 20 μ*M* 7K was also proved in terms of activation of caspase-3. In fact, the constitutive activation level of this enzyme was nearly three times increased when cells were incubated in the presence of the oxysterol (Fig. 6: A,B histograms). Addition of epicatechin fully prevented the 7K-induced upregulation of caspase-3 activity (Fig. 6: C–F histograms).

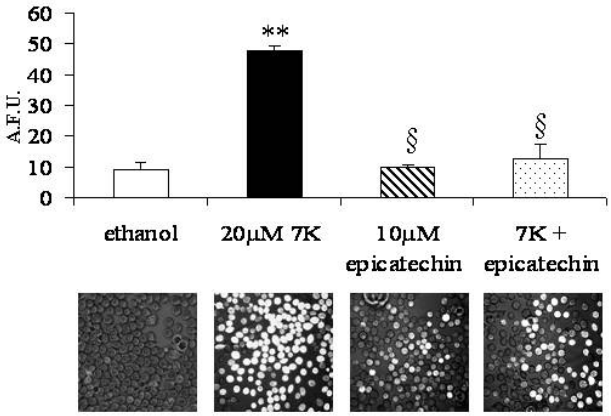

FIG. 5. Quenching effect of epicatechin on ROS overproduction NADPH-oxidase-dependent due to 7K. Cells were pre-incubated 1 h with 10 μ*M* epicatechin, then treated for 180 min with 20 μ*M* 7K. Ethanol (12.5 μ*M*) was used as solvent control. The DCF fluorescence formation was visualized by laser scanning confocal microscopy and quantified as described in Fig. 1. **Significantly different vs. ethanol ($p < 0.001$); §significantly different vs. 7K ($p < 0.001$).

DISCUSSION

7-Ketocholesterol, one of the oxysterols of major interest in pathophysiology, is consistently recovered in atherosclerotic lesions (4, Leonarduzzi *et al.*, unpublished data). This and other oxidized lipids tend to accumulate in the central core of the fibrotic plaque. The unstable plaque is indeed characterized by a relatively high amount of accumulated lipids and by an active inflammatory process. An appropriate therapy which actually succeeds in lowering the plaque's lipid content, leads to a reduced inflammatory state and to "stabilization" of the lesion (12). Oxidized lipids of the central core may exert pro-inflammatory stimuli either directly, by inducing overexpression of inflammation-related genes, or indirectly through the amplification of cell death, most likely a programmed one. Because of its pronounced toxicity against vascular cells, 7K appears to favor an inflammatory state within the atheroma mainly in an indirect way.

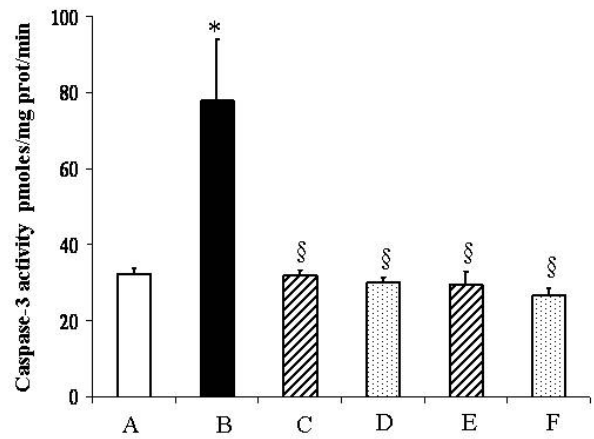

FIG. 6. Caspase-3 activity in J774A.1 macrophages incubated 18 hours with 20 μ*M* 7K; prevention by different concentrations of epicatechin. Cells were preincubated 1 h with 5 or 10 μ*M* epicatechin, then treated for 18 h with 20 μ*M* 7K. Caspase-3 specific activity was expressed as pmoles of fluorescent 7-amino-4-methylcoumarin (AMC) released/mg cell protein/min. (**A**) 21.5 μ*M* ethanol; (**B**) 20 μ*M* 7K; (**C**) 5 μ*M* epicatechin; (**D**) 10 μ*M* epicatechin; (**E**) 5 μ*M* epicatechin + 20 μ*M* 7K; (**F**) 10 μ*M* epicatechin + 20 μ*M* 7K. Data are means ± SD of three to four experiments. *Significantly different vs. ethanol ($p < 0.01$); §significantly different vs. 7K ($p < 0.01$).

As already stated in the introductory remarks, the pro-oxidant as well as the pro-apoptotic effects of 7K have already been demonstrated by this and other laboratories (2, 9). In the present report, conclusive proofs are given of the involvement of NADPH-oxidase in the intracellular oxidative imbalance provoked by biologically relevant concentrations of 7K. Very soon after macrophage challenge with 7K, definitely all cells appeared to be under oxidative stress. Such an observation was made possible by the adoption of laser confocal microscopy (Fig. 1). The almost total protection provided by two selective inhibitors of NADPH-oxidase points to the ability of 7K to potently and rapidly induce oxidative burst in the macrophage by activating this enzyme (Fig. 2). The ROS overproduction triggered by the oxysterol was also quenched strongly when the flavonoid epicatechin was present in the cell incubation reaction (Fig. 5). With regard to the suggested ability of this flavonoid to inactivate NADPH-oxidase, data here obtained treating murine macrophages with the compound in the low micromolar range (10 μ*M*) seem to exclude this event, at least in the *in vitro* model employed. In fact, constitutive DCF fluorescence remained unchanged in cell preparations treated with epicatechin only (Fig. 5).

Another interesting finding in the present research is the solid proof of a causative role for oxidative stress in apoptosis provoked by 7K. Indeed, the marked or almost complete inhibition of 7K-dependent ROS increase by the two selective inhibitors of NADPH-oxidase and by the antioxidant epicatechin allowed significant protection of the cells against 7K-induced apoptotic death, as shown both in morphological and biochemical terms (Figs. 3, 4, 6). Recent data from our laboratory showed how the rise of ROS production within J774A.1 macrophages treated with 7K was followed by activation of the mitochondrial pathway of apoptosis with eventual increase of caspase-3 activity and morphological evidence of programmed cell death (2), but validation of the likely cause-effect relationship between ROS excessive levels and apoptosis is still needed. Deeper characterization of 7K signaling to apoptosis is now in progress using macrophages as well as other vascular cell types in culture.

In conclusion, data reported here support a primary and precocious involvement of intracellular ROS overproduction in the biochemical pathway which is triggered by biologically relevant amount of 7-ketocholesterol in *in vitro* cultivated macrophages and eventually leads to cell death through apoptosis. Because this toxic oxysterol is essentially of dietary origin (9, 17), the fact that the flavonoid epicatechin was efficiently able to counteract the oxysterol's pro-apoptotic effect should stimulate further investigation about the possible role of this type of naturally occurring antioxidant in prevention of oxysterol formation during food processing and of oxy-

sterol toxicity once these compounds have been assimilated in the human body.

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ABBREVIATIONS

Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; AEBSF, 4-aminoethyl-benzenesulfonylfluoride; AMC, Asp-7-amino-4-methylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 7K, 7-ketocholesterol; LDL, low density lipoprotein; PBS, phosphate buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species.

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