The saturated medium-chain fatty acid lauric acid (LA) has been associated to certain health-promoting benefits of coconut oil intake, including the improvement of the quality of life in breast cancer patients during chemotherapy. As it concerns the potential to hamper tumor growth, LA was shown to elicit inhibitory effects only in colon cancer cells. Here, we provide novel insights regarding the molecular mechanisms through which LA triggers antiproliferative and pro-apoptotic effects in both breast and endometrial cancer cells. In particular, our results demonstrate that LA increases reactive oxygen species levels, stimulates the phosphorylation of EGFR, ERK and c-Jun and induces the expression of c-fos. In addition, our data evidence that LA via the Rho-associated kinase-mediated pathway promotes stress fiber formation, which exerts a main role in the morphological changes associated with apoptotic cell death. Next, we found that the increase of p21Cip1/WAF1 expression, which occurs upon LA exposure in a p53-independent manner, is involved in the apoptotic effects prompted by LA in both breast and endometrial cancer cells. Collectively, our findings may pave the way to better understand the anticancer action of LA, although additional studies are warranted to further corroborate its usefulness in more comprehensive therapeutic approaches.

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INTRODUCTION

Fatty acids are acyclic carboxylic acids with aliphatic tails of different lengths. Based on their carbon atom chain length, fatty acids are classified into the following three groups: short-chain fatty acids with < 6 carbon atoms, medium-chain fatty acids (MCFA) and long-chain fatty acids that contain 6–12 carbons and > 12 carbons, respectively. Fatty acids are major components of triacylglycerols, phospholipids and other complex lipids, therefore representing main contributors to dietary fat in humans. Plant oils like palm, coconut and olive oils, nuts, seeds and seed oils, cocoa butter and animal-derived fats as lard, tallow and butter, are rich of fatty acids that are important components of cell membranes and essential sources of energy. Previous studies have demonstrated that fatty acids are also involved in diverse transduction pathways, in gene transcription and relevant biological events as cell metabolism, inflammation, apoptosis and production of bioactive lipid mediators, thus contributing to multiple pathophysiological responses.

Lauric acid (LA), which is a saturated MCFA with 12 carbon atoms and the primary fatty acid of coconut oil, has been associated with certain health benefits of coconut oil intake. LA is also contained in plant oils, fruits, seeds and in breast milk. LA has been shown to elicit diverse actions in various tissues, including a potent antimicrobial property. For instance, LA and the derivative monolaurin were reported to destroy cell membranes of gram-positive bacteria and lipid-coated viruses, to interfere with main cellular responses as the activation of transduction cascades and gene transcription, to stabilize cell membranes toward the prevention of bacterial resistance. In addition, LA promoted inflammatory processes activating the nuclear factor-κB transcription factor as well as stimulating the expression of cyclooxygenase-2 and pro-inflammatory cytokines. LA was also associated with beneficial effects on the cardiovascular system due to its ability to increase the high-density lipoproteins and to reduce the blood pressure and heart rate in both normotensive and hypertensive rats. Moreover, LA prevented the prostatic hyperplasia induced by testosterone in rats, triggered apoptosis in colon cancer cells through oxidative stress and improved the sensitization of the EGFR inhibitor cetuximab in KRAS/BRAF mutated colorectal cancer cells. It is worth mentioning that the consumption of virgin coconut oil during chemotherapy improved the global quality of life in patients with breast cancer.

Here, we show for the first time that LA elicits antiproliferative and pro-apoptotic effects in breast and endometrial cancer cells promoting the generation of reactive oxygen species (ROS), the activation of transduction pathways and gene expression changes. In particular, the upregulation of the cyclin-dependent kinase inhibitor p21Cip1/WAF1 upon LA exposure was found to be required for its anticancer properties. Our findings shed new light on the molecular mechanisms through which LA induces antiproliferative and pro-apoptotic responses in both breast and endometrial cancer cells toward its usefulness in more comprehensive therapeutic approaches.

RESULTS

LA inhibits cancer cell viability

On the basis of previous findings showing that MCFAs may elicit apoptosis in certain cancer cells and considering that in our...
LA triggers ROS generation and EGFR, ERK and c-Jun phosphorylation
To evaluate the molecular mechanisms involved in the ability of LA to lower cancer cell viability, we ascertained that LA triggers the phosphorylation of EGFR, ERK and c-Jun in both SkBr3 and Ishikawa cells (Figures 2a and b). These responses were no longer observed in the presence of the EGFR inhibitor (AG) (Figures 2c and d), whereas ERK activation by LA was abolished using the MEK inhibitor (PD) and the Rho-associated kinase (ROCK) inhibitor (Y) (Figures 2c and d). These findings are in agreement with a previous report showing that Rho GTPases and their effectors as the Rho-associated protein kinase (ROCK) are key regulators of the cytoskeleton reorganization and the generation of the contractile force required for stress fiber formation.22 

LA induces gene expression changes
Then, we assessed the expression levels of well-known cell cycle regulators as the member of the AP1 transcription factor complex namely c-fos, the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21Cip1/WAF1. In both SkBr3 and Ishikawa cells, LA upregulated the mRNA expression of c-fos and p21Cip1/WAF1, without altering the levels of p53 (Figures 4a and b). In addition, LA transactivated the AP1-luc responsive collagenase promoter construct that was transiently transfected in SkBr3 and Ishikawa cells and stimulated the transcriptional activity of reporter plasmids containing the c-fos and p21Cip1/WAF1 promoter sequences (Figures 4c and d). According to the results obtained in real-time PCR, LA did not modify the p53 protein levels, whereas it increased c-fos and p21Cip1/WAF1 protein expression in both cell types (Figures 4e and f).

DISCUSSION
The present study provides novel evidence regarding the molecular mechanisms through which LA elicits antiproliferative and pro-apoptotic effects in breast and endometrial cancer cells. In particular, we have ascertained that ROS generation induced by LA triggers the activation of the EGFR/ERK/AP1 transduction pathway, leading to the upregulation of p21Cip1/WAF1 in a p53-independent manner.
Fatty acids are structural components of cellular membranes either alone or together with other molecules as phospholipids and triacylglycerides. In addition, fatty-acid oxidation occurring at the mitochondrial level plays a pivotal role in maintaining energy homeostasis during catabolic states. Nevertheless, fatty acids are currently no longer considered as mere membrane structure regulators or energy sources as they also influence diverse transduction signaling and cellular functions.

Figure 2. Lauric acid triggers rapid responses in breast and endometrial cancer cells. (a, b) Phosphorylation of EGFR, ERK1/2 and c-Jun in SkBr3 (a) and Ishikawa (b) cells treated with vehicle (−) and 100 μM LA, as indicated. (c, d) EGFR, ERK1/2 and c-Jun activation in SkBr3 (c) and Ishikawa (d) cells treated for 60 min with vehicle or 100 μM LA alone or in combination with 10 μM EGFR inhibitor AG1478 (AG), 10 μM MEK inhibitor PD98089 (PD), 1 μM JNK inhibitor SP 600125 (SP) and 10 μM ROCK inhibitor Y-27632 (Y). EGFR, ERK2 and c-Jun were used as loading controls for pEGFR, pERK1/2 and pc-Jun, respectively. Results shown are representative of at least two independent experiments.

Figure 3. ROS generation by lauric acid is involved in the activation of transduction signaling observed in breast and endometrial cancer cells. (a) ROS production determined as DCF fluorescence in SkBr3 and Ishikawa cells treated for 60 min with vehicle (−) or 100 μM LA alone or in combination with 300 μM free radical scavenger NAC, DCF fluorescence obtained in cells treated with vehicle was set as onefold induction upon which ROS levels induced by treatments were calculated. Data shown are the mean ± S.D. of three independent experiments performed in triplicate. (○) indicates P < 0.05 for cells receiving vehicle versus treatments. EGFR, ERK1/2 and c-Jun activation in SkBr3 (b) and Ishikawa (c) cells treated for 60 min with vehicle or 100 μM LA alone or in combination with 300 μM NAC. EGFR, ERK2 and c-Jun were used as loading controls for pEGFR, pERK1/2 and pc-Jun, respectively. Results shown are representative of at least two independent experiments.

Fatty acids are structural components of cellular membranes either alone or together with other molecules as phospholipids and triacylglycerides. In addition, fatty-acid oxidation occurring at the mitochondrial level plays a pivotal role in maintaining energy homeostasis during catabolic states. Nevertheless, fatty acids are currently no longer considered as mere membrane structure regulators or energy sources as they also influence diverse transduction signaling and cellular functions.
acids on human health given that the amount and type of fatty acids contained in the diet are involved in the etiopathogenesis of diabetes, cancer and cardiovascular, immunity, inflammatory, renal, hepatic diseases. In this context, coconut oil that is one of the richest sources of saturated fatty acids as LA, has attracted interest for its potential health benefits. Furthermore, coconut oil has been shown to counteract the action of stimulatory agents in colon and mammary tumors in rats and to improve the quality of life of breast cancer patients undergoing chemotherapy. As it concerns LA, Fauser and co-workers firstly demonstrated its ability to induce apoptosis in colon cancer cells through the reduction of glutathione levels and the generation of oxidative stress. In accordance with these and other observations showing that fatty acids may induce ROS generation in diverse types of cells, we have extended these findings ascertaining that LA prompts ROS-mediated apoptosis also in breast and endometrial cancer cells through the subsequent activation of relevant transduction pathways. In this respect, it is worth mentioning that the EGFR and ERK signaling are mostly referred to as regulatory pathways of cell proliferation, migration and differentiation. Nevertheless, these two main transduction mediators can also trigger apoptotic signals especially in the context of tumor cells. For instance, EGF through the cognate receptor induced the expression of the caspase 1 enzyme and p21-dependent apoptosis upon phorbol 12-myristate 13-acetate exposure in prostate cancer cells. Further extending these data, our findings have determined for the first time that LA promotes...
in breast and endometrial cancer cells the formation of stress fibers through the ROCK transduction pathway, thus suggesting that LA might be included among the activators of the Rho/ROCK signaling.

The cyclin-dependent kinase inhibitor p21 Cip1/WAF1 has an essential role in the cell cycle arrest, the transcriptional regulation, the inhibition of DNA replication, the DNA repair, the stress-induced premature senescence and the modulation of apoptosis.45–48 Numerous studies have shown that p21Cip1/WAF1 can mediate both pro- and anti-apoptotic functions depending on the type of stimulation and the cellular context.48 For instance, p21Cip1/WAF1 can prevent cells from undergoing apoptosis triggering cell cycle arrest, inactivating cyclin A/Cdk2 complexes, inhibiting the activity of procaspase 3, caspase 8 and 10, stress-activated protein kinases and apoptosis signal-regulating kinase 1.47,49 Likewise, several reports have also suggested that p21Cip1/WAF1 exerts a pro-apoptotic function under certain cellular stresses upregulating the pro-apoptotic protein Bax, activating the tumor necrosis factor family of death receptors and regulating components of the DNA repair machinery.47 It is worth mentioning that even though p21Cip1/WAF1 may represent a major p53 transcriptional target, it can promote apoptosis through both p53-dependent and independent mechanisms.47 In addition, p21Cip1/WAF1 can act as a tumor suppressor or an oncogene depending on the stimulations and the cellular context.45 In particular, various compounds eliciting an anticancer activity such as histone deacetylase inhibitors, cisplatin, phorbol 12-myristate 13-acetate and curcumin were shown to induce apoptotic cell death through the p21Cip1/WAF1 induction.40,50–54 Extending these findings, our data have ascertained that LA induces apoptosis in both breast and endometrial cancer cells upregulating the p21Cip1/WAF1 expression levels via an AP1-mediated pathway. Overall, the present results provide novel insights into the potential of LA to activate the EGFR/ERK/AP1/p21 Cip1/WAF1 transduction signaling toward antiproliferative and pro-apoptotic responses in tumor cells. Nevertheless, further experimental evidence are warranted to better define the action of LA alone or in the context of coconut oil consumption on tumor development as claimed by a current newsworthy debate.

**MATERIALS AND METHODS**

**Reagents**

LA, CA, NAC, Y-27632 (Y) and 2′,7′-dichlorofluorescin diacetate (DCFDA) were purchased from Sigma-Aldrich (Milan, Italy). Tyrphostin AG1478 (AG), PD98089 (PD), SP 600125 (SP) and UC2288 were obtained from Calbiochem (DBA, Milan, Italy). All compounds were dissolved in dimethyl sulfoxide except LA, CA, NAC and Y-27632 (Y), which were solubilized in water.

**Cell cultures**

SkBr3 breast cancer cells were obtained by ATCC, used after resuscitation and maintained in RPMI 1640 without phenol red.
supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin (Life Technologies, Milan, Italy). Ishikawa endometrial cancer cells were obtained by D Picard (University of Geneva) and maintained in MEM supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin, 2 mM L-glutamine and 1% Non-Essential Amino Acids Solution Cells (Life Technologies). Cells were switched to medium without serum the day before immunoblots and reverse transcription-PCR experiments.

Cell viability assay
Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on the conversion of MTT to MTT formazan by mitochondrial enzyme. Cells were seeded in quadruplicate in 96-well plates in regular growth medium and grown until 70–80% confluence. Cells were washed once they had attached and then treated with increasing concentrations of each compound for 48 h in regular medium supplemented with 1% FBS. Relative cell viability was determined by MTT assay according to the manufacturer’s protocol (Sigma-Aldrich). Mean absorbance of cells receiving vehicle (−) was set as onefold induction upon which the mean absorbance of treatments was calculated.

Gene expression studies
Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc, Milan, Italy). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems). For c-fos, p53, p21Cip1/WAF1 and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-GGAGCGGGCTGTCT-3′ (c-fos reverse); 5′-GGACTTGGAGGAGCTCTCT-3′ (p53 reverse); 5′-GCTTCATGGCAGCTAATTC-3′ (p21 forward); 5′-CTGTGCTCACTTCAGGGTCA-3′ (p53 forward), 5′-GGCATTCTGGGAGCTTCATCT-3′ (p21 reverse); 5′-GGCATTCTGGGAGCTCTCT-3′ (p53 reverse); 5′-GGGAGAGCTGTGCTCTCT-3′ (c-fos forward)

Figure 6. Lauric acid promotes apoptosis in cancer cells. SkBr3 and Ishikawa cells were treated for 4 h with vehicle (−) (a, d) or 100 μM LA alone (b, e) or in combination with 10 μM ROCK inhibitor Y-27632 (Y) (c, f) and subjected to phalloidin staining to visualize F-actin. (g, h) Enlarged details of stress fibers shown in b and e, respectively. White arrows indicate stress fibers. Images shown are representative of 30 random fields obtained in three independent experiments. Scale bar: 12.5 μm.
Lauric acid induces apoptotic cell death. (a, d) TdT-mediated dUTP nick-end-labeling (TUNEL) staining (green) in SkBr3 (a) and Ishikawa (d) cells treated for 18 h with vehicle (–) or 100 μM LA alone or in combination with 300 μM free radical scavenger NAC and 10 μM p21Cip1/WAF1 inhibitor UC2288, as indicated. Nuclei were stained by propidium iodide (PI, red). Magnification is indicated by bars (100 μm). Each experiment shown is representative of 20 random fields observed. (b, e) Bar graphs represent the percentage of TUNEL-positive cells upon treatments versus vehicle. Values are the mean of three independent experiments. (*) indicates \( P < 0.05 \) for cells receiving vehicle versus treatments. (c, f) Efficacy of p21Cip1/WAF1 downregulation by UC2288. β-actin was used as a loading control. Results shown are representative of at least two independent experiments.
Chromatin immunoprecipitation assay
Cells were grown in 10-cm dishes to 70–80% confluence, shifted to serum-free medium for 24 h and then treated with vehicle (–) or 100 μM LA for 4 h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleaned with salmon DNA/protein A-agarose (Upstate Biotechnology, Inc., Lake Placid, NY, USA) and immunoprecipitated with anti c-fos (H-125) antibody or non-specific IgG (Santa Cruz Biotechnology, DBA, Milan, Italy). Pellets were washed, eluted with a buffer consisting of 1% SDS and 0.1 mol/l NaHCO₃, and digested with proteinase K. DNA was obtained by phenol/chloroform extraction and precipitated with ethanol. A 4 μl volume of each sample was used to replicate a PCR containing an AP-1 site from the pcDNA3.1(+) vector.

Western blot analysis
Cells were grown in 10-cm dishes, exposed to ligands, and then lysed as previously described.57 Equal amounts of whole-protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Sigma-Aldrich, Milan, Italy), which were probed with primary antibodies against pEGFR Tyr 1173, EGFR (1005), phosphorylated ERK1/2 (E-4), ERK2 (C-14), p-c-Jun S73, c-Jun (Santa Cruz Biotechnology) and then revealed using the chemiluminescent substrate for western blotting Westar Nova 2.0 (Cyanagen, Biogeneerbica, Catania, Italy).

ROS production
The non-fluorescent DCFDA probe, which becomes highly fluorescent on reaction with ROS, was used to evaluate intracellular ROS production. In brief, cells (2 × 10⁵) were incubated with 10 μM DCFDA (Sigma-Aldrich) at 37 °C for 30 min, washed with PBS and then exposed to treatments, as indicated. Cells were washed with PBS and the fluorescent intensity of DCF was measured (excitation at 485 nm and emission at 530 nm).

Phalloidin staining
Cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, washed briefly with PBS, and then incubated with Phalloidin-Fluorescent Conjugate (Santa Cruz Biotechnology) and visualized with the Olympus BX41 microscope and the images were taken with CSV1.14 software using a CAM XC-30 for images acquisition (Olympus Europa, Hamburg, Germany).

TUNEL assay
Cell apoptosis was determined by TUNEL assay, conducted using DeadEnd Fluorometric TUNEL System (Promega) and performed according to the manufacturer’s instructions. In brief, cells were treated for 18 h, as indicated, then fix in freshly prepared 4% paraformaldehyde solution in PBS (pH 7.4) for 25 min at 4 °C. After fixation, cells were permeabilized in 0.2% Triton X-100 solution in PBS for 5 min. After washing twice with washing buffer for 5 min, the cells were covered with equilibration buffer at room temperature for 5–10 min. The labeling reaction was performed using terminal deoxynucleotidyl transferase end-labeling TdT and fluorescein-dUTP cocktail for each sample and incubated for 1 h at 37 °C where TdT catalyses the binding of fluorescein-dUTP to free 3’OH ends in the nicked DNA. After rinsing, cells were washed with 2 × SSC solution buffer and subsequently incubated with propidium iodide (Sigma-Aldrich) to stain nuclei and analyzed using the Cytation 3 Cell Imaging Multimode Reader (BioTek, Winooski, VT, USA).

Statistical analysis
Statistical analysis was done using ANOVA followed by Newman–Keuls’ testing to determine differences in means. P < 0.05 was considered as statistically significant.

COMPETING INTERESTS
The authors declare no conflict of interest.

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Lauric acid triggers apoptosis in cancer cells

R Lappano et al

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