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Blockade of Oxidized LDL-Triggered Endothelial Apoptosis by Quercetin and Rutin through Differential Signaling Pathways Involving JAK2

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Oxidized LDL is highly atherogenic, as it stimulates foam cell formation and promotes inflammatory and thrombotic processes. The present study elucidated whether the antioxidants quercetin and its rutinoside rutin exert antiapoptosis in endothelial cells exposed to Cu^{2+}-oxidized LDL. Quercetin and rutin inhibited the oxidized LDL-induced endothelial toxicity at nontoxic doses of ≤ 25 μM with an inhibition of intracellular oxidant accumulation. These effects accompanied disappearance of apoptotic bodies and suppression of caspase-3 activation. Additionally, condensed nuclei vanished in oxidized LDL-exposed cells treated with quercetin and rutin. This study further explored whether such effects were achieved by redox manipulation via JAK2-STAT3-responsive death/survival signaling pathways involving multiple MAPK. Unlike quercetin, rutin blocked the activation of oxidized LDL-induced JNK and p38 MAPK as well as the upstream ASK1 phosphorylation. Quercetin dose-dependently attenuated the JAK2 phosphorylation evoked by oxidized LDL, whereas rutin abolished the JAK signaling accompanying nuclear transactivation of STAT3 and enhanced the JAK activity-inhibiting SOCS3 expression. Conversely, oxidized LDL-induced IL-6 release was minimal for the JAK2 activation, although this effect was counteracted by quercetin and rutin. These results suggest that quercetin and rutin inhibit Cu^{2+}-oxidized LDL-induced endothelial apoptosis through modulating JAK2-STAT3 pathways and that rutin may modulate a signaling crosstalk between JAK2 and MAPK.

KEYWORDS: Endothelial apoptosis; JAK2; MAPK; oxidized LDL; quercetin; rutin; STAT3

INTRODUCTION

Low-density lipoprotein (LDL) is oxidatively modified in the arterial wall and causally involved in atherosclerosis (*1*, *2*), which in turn results in lipid accumulation, focal necrosis, connective tissue proliferation due to chronic inflammation, and other subparenchymal events (*3*). Oxidative modification of LDL occurs in cell-free and cell-containing systems with transition metals such as copper (Cu^{2+}) or inorganic oxidants such as H_2O_2 (*4*). The resultant oxidized LDL may promote atherosclerosis through multiple mechanisms pertaining to its toxicity toward endothelial cells and macrophage cells (*4*). Like cells involved in the atherosclerotic plaque, vascular cells undergo apoptosis upon treatment with oxidized LDL (*5*). Micromolar oxysterols present in oxidized LDL may induce apoptosis through mitochondrial pathways (*6*). It has been shown that oxidized LDL induces endothelial apoptosis by producing lipid peroxidative products (*7*). Additionally, oxidative stress activates death signal cascades leading to apoptosis through redox state-related signaling pathways (*8*). Thus, antioxidants such as vitamin C blunting production of reactive oxygen species (ROS) prevent apoptosis (*9*). However, the underlying mechanisms by which antioxidants prevent apoptosis evoked by diverse stimulators remain to be elucidated.

Epidemiologic studies showed that a high consumption of polyphenols alleviates the risk of cardiovascular diseases (*10*), and this phenomenon arises from their antioxidant capacity (*11*). The antioxidant kaempferol diminished the apoptosis induced by a component of oxidized LDL (*12*). Berberine attenuated oxidized LDL-induced apoptosis and ROS generation, suggesting that the natural constituent of the plant berberis may protect against oxidized LDL-induced endothelial dysfunction (*11*). Oxidized lipids triggered ROS overproduction and impairment

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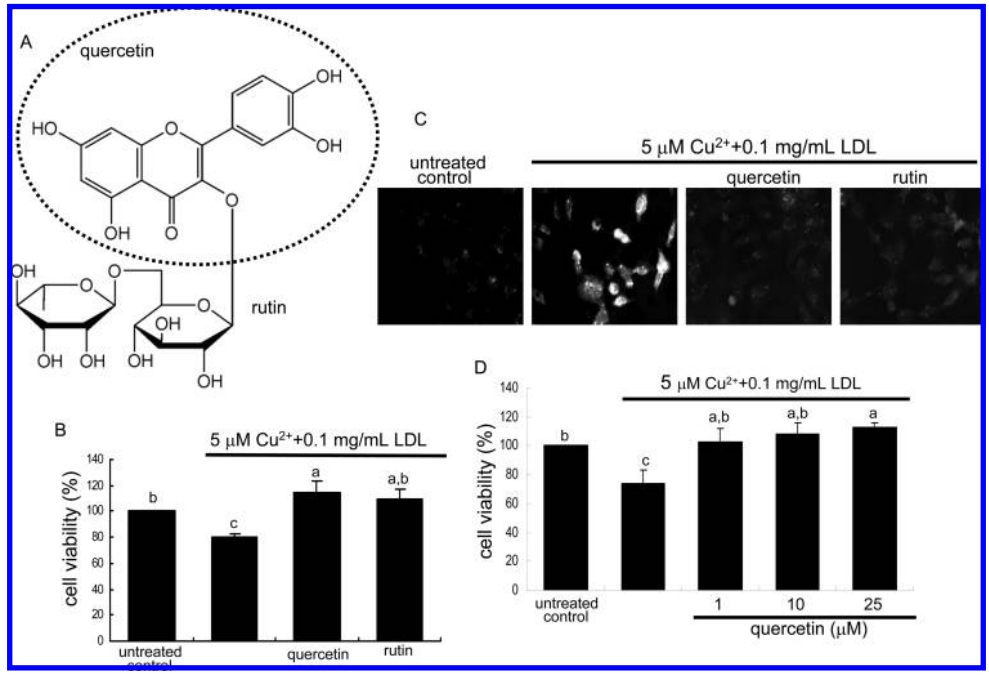

Figure 1. Structures of quercetin and rutin (**A**) and cell viability (**B**, **D**) and ROS production (**C**) of quercetin- or rutin-treated HUVEC exposed to Cu^{2+}-oxidized LDL. Endothelial cells were pretreated with 25 μM quercetin or rutin and exposed to 0.1 mg/mL LDL-cholesterol in the absence and presence of 5 μM Cu^{2+} for 24 h (**B**). ROS production following a treatment with 25 μM quercetin or rutin in the presence of 5 μM Cu^{2+}-oxidized LDL was measured by DCFH fluorescence (**C**). Representative cell images were obtained using a fluorescence microscopy (four separate experiments). Magnification: $\times$200. Quercetin was treated at nontoxic doses of 1−25 μM to examine its dose−responses (**D**). Cell viability to oxidized LDL toxicity was determined by MTT assay. The values are means $\pm$ SEM ($n = 4$) and expressed as percent cell survival relative to untreated control cells.

of antioxidant systems, which rendered intestinal cells to be apoptotic-prone, which effects were fully counteracted by dietary phenolics of tyrosol and protocatechuic acid (*13*).

Numerous reports have revealed that ROS may instigate apoptosis via activation of mitogen-activated protein kinase (MAPK) in various oxidative states (*14*, *15*). Hyperoxia, by virtue of activating NADPH oxidase, generated ROS and provoked lung epithelial cell death through activating extracellular signal-regulated kinase (ERK1/2) (*14*). The antioxidant *N*-acetylcysteine (NAC) inhibited activation of c-Jun N-terminal kinase (JNK) and p38 MAPK and activity of redox-sensitive apoptotic genes (*15*). In addition, Janus kinase (JAK)-signal transducers and activators of the transcription (STAT) pathway are known to play a role in myocardial injury including myocardial infarction and oxidative damage (*16*). Cu^{2+}-oxidized LDL or cell-mediated oxidized LDL induced tyrosine phosphorylation of JAK2, STAT1, and STAT3 via oxidative stress by means of its lipid peroxidation products (*17*). Moreover, STAT1/3-dependent mechanisms participated in the transduction of death/survival signals (*18*).

On the basis of the literature evidence that polyphenols inhibit oxidized LDL-induced endothelial apoptosis through exerting their antioxidant activity (*7*), we hypothesized that polyphenol-triggered redox manipulations block oxidized LDL-induced apoptosis by influencing JAK2-STAT3-responsive signaling pathways and MAPK-dependent mechanisms. To test this hypothesis, this study elucidated action mechanisms of dietary antioxidants, quercetin abundant in onions, and quercetin-3-rutinoside rutin found in buckwheat (**Figure 1A**) for the protection of human umbilical vein endothelial cells (HUVEC) against Cu^{2+}-oxidized LDL-induced endothelial apoptosis.

MATERIALS AND METHODS

Materials. Polyphenolic flavonoids (flavonols of quercetin and its rutinoside rutin), M199 medium chemicals, cell growth supplements (human epidermal growth factor and hydrocortisone), and 2′,7′-dichlorodihydrofluorecein diacetate (DCFH) were obtained from Sigma-Aldrich Chemical (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Fetal bovine serum (FBS), trypsin−EDTA, and penicillin−streptomycin were purchased from Lonza (Walkersvillle, MD). Collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). Antibodies of human caspase-3, human phospho-ASK1, human phospho-JNK, human phospho-p38 MAPK, human phospho-JAK2, human suppressor of cytokine signaling 3 (SOCS3), and human phospho-STAT3 were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was supplied from Jackson ImmunoResearch Laboratory (West Grove, PA). Quercetin and rutin were dissolved in dimethyl sulfoxide (DMSO) for live culture with cells; a final culture concentration of DMSO was $\leq$0.1%.

Preparation of Human Plasma LDL. Human plasma LDL was isolated by discontinuous density gradient ultracentrifugation as previously described (*7*). LDL fractions obtained from pooled human normolipidemic blood were dialyzed overnight against 0.154 M NaCl and 0.01% EDTA (pH 7.4) at 4 °C and used within 4 weeks. Plasma LDL protein was determined according to the Lowry method (*19*), and concentrations of triacylglycerol, total cholesterol, and phospholipid were measured using commercial kits (Asan Pharmaceutical Co.). The contents of total protein, triacylglycerol, total cholesterol, and phospholipid in the prepared LDL fraction were all within the appropriate ranges.

LDL oxidation was validated by analyzing its electrophoretic mobility and lipid peroxidation. For the electrophoretic mobility test, aliquots of prepared LDL were run on 0.8% agarose electrophoresis gels in barbital buffer (pH 8.6). The gel was immediately fixed in 5% trichloroacetic acid solution and rinsed in 70% ethanol. Photographs of gels were obtained using Polaroid type 667 positive/negative film (Polaroid Co., Wayland, MA). In addition, production of thiobarbituric acid reactive substances (TBARS) was measured as an indicator of lipid peroxidation (*20*).

Primary Culture of Endothelial Cells. HUVEC were isolated by an enzymatic technique using collagenase as described elsewhere (*21*). Cells were cultured in 25 μM HEPES-buffered M199 containing 10%

FBS, 2 mM glutamine, 0.75 mg/mL human epidermal growth factor, and 75 μg/mL hydrocortisone at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were identified by verifying their cobblestone morphology and uptake of acetylated LDL fluorescently labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes Inc., Eugene, OR).

Cells were pretreated with 25 μM quercetin and rutin and exposed to 0.1 mg/mL LDL-cholesterol in the absence and presence of Cu^{2+} for 24 h to induce endothelial apoptosis. After the incubation with Cu^{2+}-oxidized LDL, a 3-(4,5-dimetylthiazolyl)diphenyl tetrazolium bromide (MTT) assay was conducted to quantitative cellular viability (*22*). HUVEC were incubated in a fresh medium containing 1 mg/mL MTT for 3 h at 37 °C. The purple formazan product was dissolved in 0.5 mL of isopropanol with gentle shaking. Absorbance of formazan was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm.

ROS Detection. Oxidant generation of HUVEC was measured according to a method with a minor modification (*22*). On the basis of an oxidant conversion of DCFH to the fluorescent 2′,7′-dichlorofluorecein (DCF), ROS was for measuring produce. Cells were challenged with Cu^{2+}-oxidized LDL and incubated for 30 min with 10 μM DCFH in prewarmed M199 (+2% FBS). Fluorescent images were taken by a fluorescent microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan).

Nuclear Staining. After cells were fixed with 4% formaldehyde for 20 min and washed with ice-cold phosphate-buffered saline (PBS), Hoechst 33258 (Sigma-Aldrich Co.) was added at a final concentration of 1 μg/mL for 15 min to allow uptake and equilibration before microscopic observation. Cells containing fragmented or condensed nuclei were considered to be apoptotic, whereas those containing diffuse and irregular nuclei were considered to be necrotic. Nuclear morphology was examined using a fluorescent microscope (Carl Zeiss Co., Oberkochen, Germany).

Western Blot Analysis. Western blot analysis was performed using whole cell extracts from HUVEC as previously described (*22*). Equal amounts of total protein of cell lysates were subjected to electrophoresis on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in 0.5 M Tris-HCl (pH 7.5) buffer containing 1.5 M NaCl and 1% Tween 20 (TBS-T) with 3% bovine serum albumin for 3 h. The membrane was incubated overnight at 4 °C with a primary polyclonal rabbit antibody (anticleaved caspase-3, antiphospho-ASK1, antiphospho-JNK, antiphospho-p38 MAPK, antiphospho-JAK2, anti-SOCS3 and antiphospho-STAT3). After three washes with TBS-T, the membrane was incubated for 1 h with a goat anti-rabbit IgG conjugated to horseradish peroxidase. The individual protein level was determined using Super Signal West Pico chemiluminescsnce detection reagents (Pierce Biotechnology, Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with polyclonal mouse β-actin antibody (Sigma-Aldrich Chemical) was also performed for the comparative controls.

Measurement of Caspase-3 Activity. Caspase-3 activity was detected by a colorimetric assay kit (Clontech Laboratories., Mountain View, CA). This assay was based on the detection of colored *p*-nitroanaline (*p*NA) molecule following a cleavage from DEVD-*p*NA labeled substrate. The optical density was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Nuclear Extract Preparation. Nuclear protein extracts from HUVEC were prepared using a detergent lysis procedure to assay the DNA binding activity of STAT3 (*21*). Cells lysed in a buffer of 10 mM HEPES (pH 7.9), 1 mM EDTA,1 mM dithiothreitol, 0.5% Nonidet P40, 0.4 mM phenylmethanesulfonyl fluoride, 0.01 ng/mL leupeptin, and 0.02 ng/mL aprotinin were incubated on ice for 30 min. Proteins were extracted from nuclear pellets by an incubation with a high-salt buffer containing 420 mM NaCl, 1 mM EDTA, 20 mM HEPES (pH 7.9), 20% glycerol, 1 mM dithiothreitol, 0.4 mM phenylmethanesulfonyl fluoride, 0.01 ng/mL leupeptin, and 0.02 ng/mL aprotinin with vigorous shaking. The nuclear debris was pelleted by a centrifugation at 2000 g for 30 min, and the supernatant was stored at −20 °C. For the determination of STAT3 localization, Western blot analysis was conducted with nuclear protein extracts using human STAT3 primary antibody as described above.

Transcriptional Reporter Gene Assay. To determine the STAT3 promoter activity, a promoter-report construct with luciferase gene driven by the translucent STAT3 reporter vector (Panomics Inc., Fremont, CA) and STAT3 siRNA (Santa Cruz Biotechnology) were employed. Nucleofection of HUVEC was performed for the gene delivery according to the optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). Briefly, reporter constructs were made by replacing *Nhe*I and *Bgl*II fragments of pSTAT3-TA-Luc (pSTAT3-TA-Luc). HUVEC were pelleted, gently resuspended in the Amaxa cuvette with 100 μL of nucleofector solution mixed with 3 μg of STAT3 reporter vector or 3 μM STAT3 siRNA, and pulsed in the nucleofector device with the program A-34. Cells were immediately transferred into prewarmed fresh medium in 12-well plates. After 24 h of nucleofection, transfected cells were exposed to Cu^{2+}-oxidized LDL in the presence of quercetin and rutin for 2 h. At 2 h post-transfection the cells were washed with PBS, and 100 μL of cell lysis reagent (Promega Biosciences, Madison, WI) was added. Promoter activity of HUVEC nucleofected with a luciferase-harboring pSTAT3−promoter construct was luciferase-assayed. For the measurement of luciferase activity (*7*), 5 μL of lysate mixed with 20 μL of assay reagent (Promega Biosciences) was assayed using a luminometer (Lumat LB 9501, Berthold, Germany).

Enzyme-Linked Immunoadsorbent Assay (ELISA). Cell supernatants collected from cultures were stored at −20 °C until cytokine measurements by ELISA. Interleukin (IL)-6 was measured in duplicate with a human IL-6 ELISA QuantiGlo Kit (R&D systems, Minneapolis, MN) using a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

Data Analyses. The results are presented as mean ± SEM. Statistical analyses were conducted using the Statistical Analysis statistical software package from SAS Institute Inc. (Cary, NC). One-way ANOVA was used to determine differential effects of quercetin and rutin on redox-sensitive signaling pathways mediating apoptosis of 5 μM Cu^{2+}-oxidized LDL-exposed HUVEC. Differences among treatment groups were analyzed with Duncan's multiple-range test and were considered to be significant at $p < 0.05$.

RESULTS

Blockade of Oxidized LDL-Induced Endothelial Apoptosis by Quercetin and Rutin. Electrophoretic mobility test revealed that LDL underwent a substantial oxidative modification in the presence of the nontoxic dose of 5 μM Cu^{2+} (*7*). During 24 h of incubation with 0.1 mg/mL LDL-cholesterol in the presence of 5 μM Cu^{2+}, the HUVEC viability decreased by ≈20−30%, evidenced by MTT assay (**Figure 1B**). This cytotoxicity appeared to be as a consequence of a several-fold increase in TBARS (*20*). Treatment of HUVEC with 25 μM quercetin and rutin abolished the toxicity of 0.1 mg/mL LDL in the presence of 5 μM Cu^{2+} (**Figure 1B**). DCF staining revealed that Cu^{2+}-oxidized LDL-exposed cells elevated intracellular ROS formation, which was counteracted by 25 μM quercetin and rutin (**Figure 1C**). In addition, quercetin at doses of ≥1 μM fully prevented Cu^{2+}-oxidized LDL-elicited HUVEC toxicity. Rutin and quercetin-3 glucoside substantially mitigated the rate of oxidized LDL-induced cell death even at the dose of 0.1 μM (data not shown). Accordingly, submicromolar quercetin and its derivatives blocked endothelial cell injury caused by Cu^{2+}-oxidized LDL, which may be at least attributed to an accumulation of intracellular oxidants.

To determine whether the oxidized LDL-elicited HUVEC toxicity was apoptotic, nuclear morphology and caspase-3 activation were examined. Fluorescent staining with Hoechst 33258 dye showed that Cu^{2+}-oxidized LDL caused nuclear condensation and the appearance of apoptotic bodies (**Figure 2A**). In addition, the cellular level of cleaved caspase-3 was enhanced in Cu^{2+}-oxidized LDL-exposed cells (**Figure 2B**), and its activity expressed as DEVDase-specific activity was also

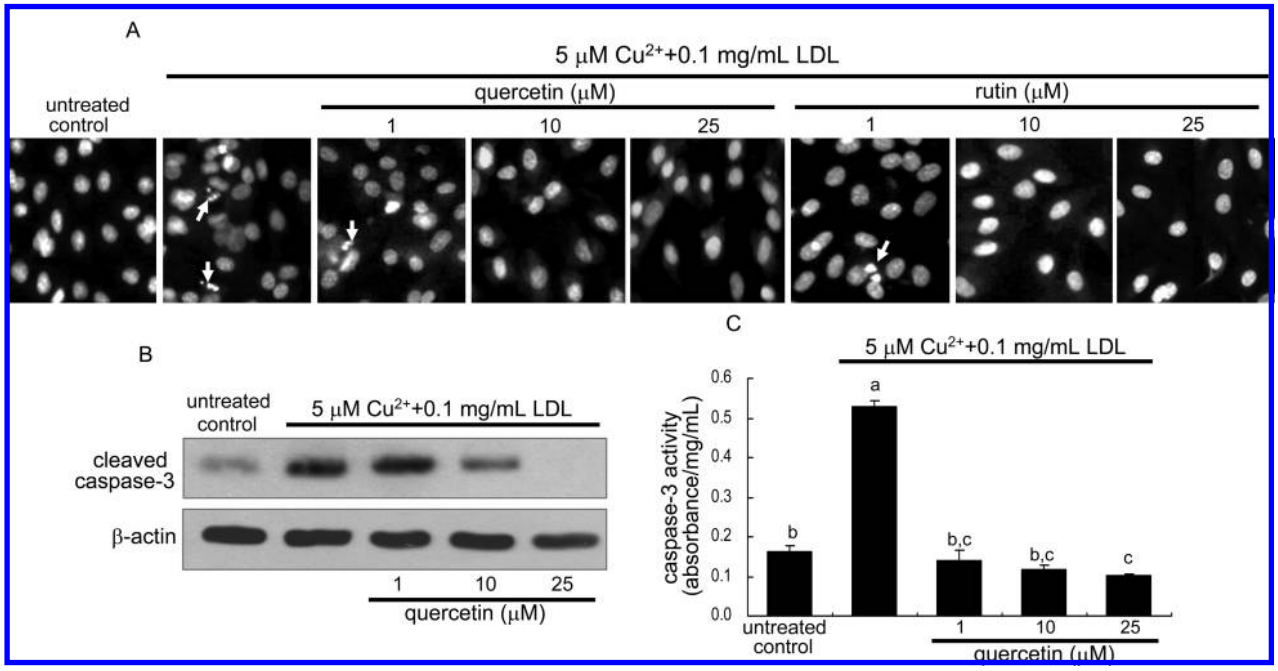

Figure 2. Nuclear morphology (**A**) and caspase-3 activation (**B**, **C**) in quercetin- or rutin-treated HUVEC exposed to Cu^{2+}-oxidized LDL. Endothelial cells were pretreated with 1−25 μM quercetin or rutin and exposed to 0.1 mg/mL LDL-cholesterol in the absence and presence of 5 μM Cu^{2+}. Nuclear morphology of HUVEC was examined by staining with Hoechst 33258 (**A**). Cu^{2+}-oxidized LDL caused nuclear condensation and the appearance of apoptotic bodies (arrows). This is representative of five independent slides. Magnification: ×200. Total cell protein extracts were immunoblotted with a primary antibody against cleaved caspase-3 and β-actin as an internal control (**B**). Bands represent three independent experiments. DEVDase-specific activity of cell extracts for the measurement of caspase-3 activity was determined (**C**). Values are means ± SEM ($n = 3$), and means without a common letter differ, $P < 0.05$.

significantly increased (**Figure 2C**). When HUVEC were pretreated with ≥10 μM quercetin and rutin, apoptotic bodies disappeared and HUVEC were devoid of nuclear condensation (**Figure 2A**). The enhanced cleavage and activity of caspase-3, a key enzyme in the apoptotic signaling process, were markedly attenuated in ≥1 μM quercetin-treated cells exposed to Cu^{2+}-oxidized LDL for 18−24 h. Thus, culture with submicromolar quercetin and rutin fully protected HUVEC against oxidized LDL-induced apoptosis through the inhibition of caspase-3 activation.

Differential Inhibition of MAPK in Response to Quercetin and Rutin. Oxidized LDL is shown to activate MAPK in the signaling pathway leading to apoptosis (*7*). This study attempted to determine whether quercetin and rutin block oxidized LDL-exposed endothelial apoptosis through interrupting signaling cascades of JNK and p38 MAPK. The treatment of cells with oxidized LDL instigated the JNK phosphorylation within 30 min, which was attenuated by rutin in a dose-dependent manner (**Figure 3A**). In contrast, quercetin failed to exert such effects with a minimal inhibition of the activation of ASK1, a redox-sensitive MAPK regulating p38 MAPK and JNK cascades (**Figure 3B**,**C**). Rutin even at the dose of 1 μM abolished Cu^{2+}-oxidized LDL-induced activation of ASK1 (**Figure 3C**).

Consistent with ASK-1 activation, Cu^{2+}-oxidized LDL rapidly elevated the phosphorylated levels of p38 MAPK within 30 min (data not shown), which declined only in 25 μM quercetin-treated cells (**Figure 4A**). In contrast, rutin nearly completely dampened p38 MAPK activation in cells exposed to Cu^{2+}-oxidized LDL (**Figure 4B**). Following the addition of SB203580, a p38 MAPK inhibitor, to oxidized LDL-exposed cells, the phosphorylation of p38 MAPK was considerably mitigated and the cell viability was restored (**Figure 4A**,**C**). These results indicate that rutin blocks Cu^{2+}-oxidized LDL-induced activation

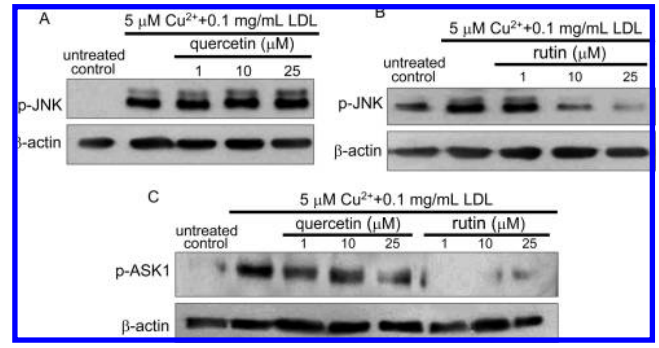

Figure 3. Western blot data showing the effects of quercetin and rutin on phosphorylation of JNK (**A**, **B**) and ASK1 (**C**) in 5 μM Cu^{2+}-oxdized LDL-exposed HUVEC. Cells were treated with 1−25 μM quercetin or rutin in the presence of 0.1 mg/mL LDL-cholesterol and 5 μM Cu^{2+} for 30 min. Total cell protein extracts were immunoblotted with a primary antibody against phosphorylated JNK and ASK1. β-Actin protein was used as an internal control. Bands represent three independent experiments.

of JNK and p38 MAPK and that oxidized LDL-generated ROS acts as mediators of the ASK1-MAPK pathway and culminates in apoptosis.

Influence of Quercetin and Rutin on JAK2-STAT3 Signaling. The present study also investigated whether the involvement of the JAK-STAT pathway is responsible for endothelial apoptosis induced by oxidized LDL and whether the antiapoptotic effects of quercetin and rutin entail JAK-STAT-dependent mechanisms. The JAK2 phosphorylation was rapidly induced by Cu^{2+}-oxidized LDL within 30 min, and this activation was sustained for up to 2 h (**Figure 5A**). The elevated phosphorylation of JAK2, upstream of STAT, was dose-dependently attenuated by quercetin and completely abolished by ≥1 μM rutin (**Figure 5B**). Conversely, quercetin and rutin promoted

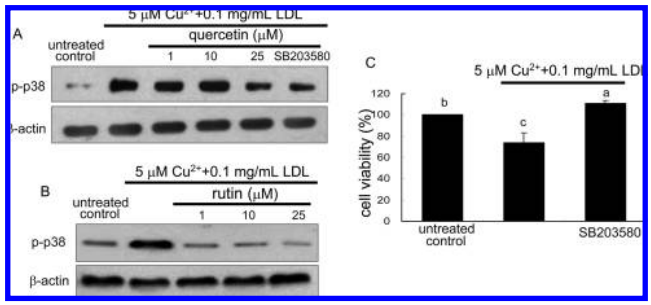

Figure 4. Inhibition of p38 MAPK phosphorylation in 5 μM Cu^{2+}-oxidized LDL-exposed endothelial cells treated with quercetin (**A**) and rutin (**B**). Cells were treated with 1−25 μM quercetin and rutin in the presence of 5 μM Cu^{2+}-oxidized LDL for 30 min. Total cell protein extracts were electrophoresed on 10% SDS-PAGE, followed by Western blot analysis with a primary antibody against phosphorylated p38 MAPK. β-Actin was used as an internal control. Respective representative blots are typical of four separate experiments. Cell viability to oxidized LDL toxicity was determined by MTT assay. Endothelial cells were pretreated with 1 μM p38 MAPK inhibitor SB203580 and exposed to 0.1 mg/mL LDL-cholesterol in the absence and presence of 5 μM Cu^{2+} for 24 h (**C**). The values are means ± SEM ($n = 4$) and expressed as percent cell survival relative to untreated control cells.

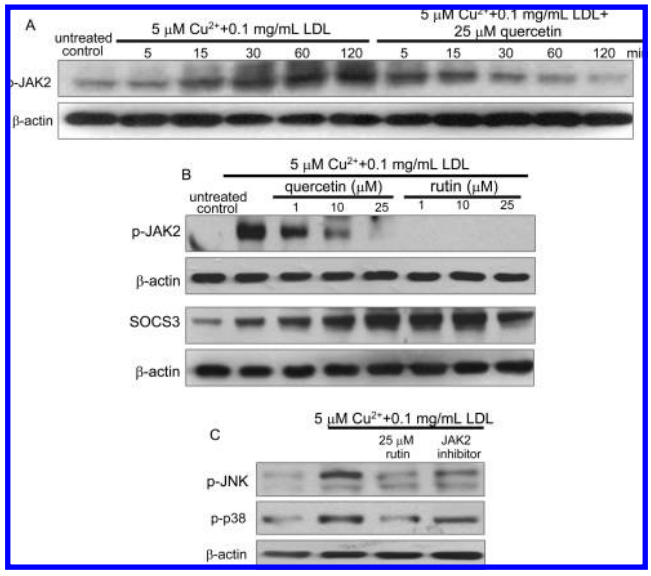

Figure 5. Time course inhibition of JAK2 phosphorylation (**A**) and inhibitory effects of 5 μM Cu^{2+}-oxidized LDL on JAK2 activation and SOCS3 expression (**B**) in quercetin- and rutin-pretreated endothelial cells. HUVEC were treated with 1−25 μM quercetin and rutin in the presence of 5 μM Cu^{2+} and 0.1 mg/mL LDL for 30 min. Cu^{2+}-oxidized LDL-induced activation of JNK and p38 MAPK was blocked by 25 μM rutin and 20 μM JAK2 inhibitor within 2 h (**C**). Total cell protein extracts were subjected to Western blot analysis with a primary antibody against phosphorylated JAK2, SOCS3, JNK, or p38 MAPK. β-Actin was used as an internal control. Respective bands represent three separate experiments.

in opposite fashions the expression of SOCS3, an inducible inhibitor that negatively regulates STAT signaling pathways (**Figure 5B**). In addition, the JAK2 inhibitor suppressed JNK activation, implying that JNK phosphorylation may be greatly dependent on JAK2 signaling (**Figure 5C**). It is assumed that rutin may modulate crosstalk between the signaling of MAPK and JAK2 instigated by oxidized LDL.

Oxidized LDL also immediately augmented activation of STAT3, the JAK downstream effector, and the phosphorylation remained high for up to 6 h (**Figure 6A**). Quercetin and rutin at 1−25 μM blocked the phosphorylation of STAT3, suggesting that these flavonoids may be antagonists to this STAT3 induction of HUVEC in response to Cu^{2+}-oxidized LDL (**Figure 6B**). Nuclear translocation of STAT3 promoted by oxidized LDL was also inhibited by adding quercetin and rutin. It should be noted that rutin attenuated oxidized LDL-induced endothelial STAT3 transactivation to a greater extent than did quercetin. Likewise, oxidized LDL-elevated promoter activity of STAT3 was markedly reversed by 25 μM quercetin and rutin, together with STAT3 siRNA specifically knocking down the endothelial expression of STAT3 (**Figure 6C**). These results indicate that the JAK-STAT pathway was triggered in oxidized LDL-mediated endothelial apoptosis, which was reversed by both quercetin and rutin.

Influence of Oxidized LDL on IL-6 Signaling. This study hypothesized that Cu^{2+}-oxidized LDL promoted IL-6 secretion and in turn activated the JAK-STAT pathway in HUVEC. Oxidized LDL secreted IL-6 by ≈400 pg/mL, and the augmented release was reversed by 25 μM quercetin and rutin (**Figure 7A**). However, the activation of JAK2 signaling promoted by 10 ng/mL IL-6 failed to occur at the very IL-6 concentration secreted in Cu^{2+}-oxidized LDL-exposed endothelial cells (**Figure 7B**). This implies that oxidized LDL promoted JAK2 activation in a direct way, which sequentially activated STAT3 signaling and MAPK-dependent mechanisms leading to endothelial apoptosis.

DISCUSSION

It is assumed that endothelial dysfunction or activation elicited by oxidized LDL is the key step in the initiation of atherosclerosis (*2*). Mildly oxidized LDL prevalent in earlier stages of atherosclerotic lesions is capable of inducing apoptosis in vascular cells (*7*, *23*). Oxidized LDL induced apoptosis in murine RAW 264.7 macrophages through promoting Fas/FasL-dependent activation of caspase-8 and the mitochondria pathway (*5*). Bcl-2 overexpression prevented bax translocation, whereas it failed to prevent ROS generation, indicating that ROS is an upstream signal for inducing mitochondrial apoptotic damages. This study attempted to identify multiple redox-sensitive signaling pathways induced by oxidized LDL leading to ROS generation. Generators of ROS causing LDL oxidation in macrophages include myeloperoxidase-mediated respiratory burst and lipid raft-associated NADPH-oxidase (*24*). Recently, it was reported that oxidized LDL induced ROS generation in endothelial cells through the lectin-like oxidized LDL receptor-1 and the cytosolic component of NAD(P)H oxidase activator of p22(phox) (*25*). Resveratrol and tyrosol, representative components of wine and olive oil, reverted H_2O_2 production induced by oxidized LDL, and such effects were not as a consequence of these compounds deterring the receptor binding of oxidized LDL. However, the molecular causes for oxidized LDL to induce oxidative damage and dysfunction of arterial endothelium remain uncertain.

ROS may instigate apoptosis via activation of MAPK in various oxidative states (*14*). It has been previously shown that Cu^{2+}-oxidized LDL induces endothelial apoptosis by activating JNK and p38 MAPK signaling (*7*). Hyperoxia generating ROS resulted in lung epithelial cell death through the activation of ERK1/2. In contrast, oxidized LDL stimulated smooth muscle cell proliferation via the phosphorylation of ERK1/2 and JNK and the activation of AP-1 and NF-κB, which was attenuated by overexpression of antioxidant enzymes (*26*). In addition, low concentrations of oxidized LDL blunted apoptosis of staurosporine-induced monocytic cells via the ERK signaling pathway

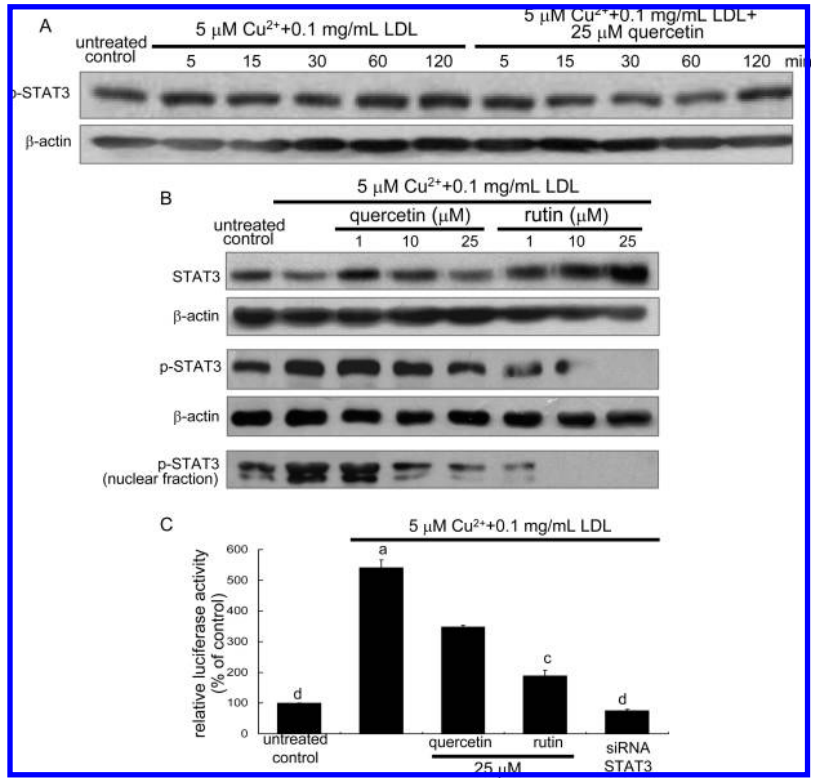

Figure 6. Inhibition of Cu^{2+}-oxidized LDL-induced STAT3 phosphorylation by quercetin and rutin (**A**, **B**) and promoter activity in transfected endothelial cells treated with quercetin and rutin (**C**). HUVEC were treated with 1−25 μM quercetin and rutin in the presence of 5 μM Cu^{2+} and 0.1 mg/mL LDL for 30 min. For Western blot analysis, total cell protein extracts and nuclear proteins were immunoblotted with a primary antibody against STAT3, phosphorylated STAT3, or β-actin as an internal control. Bands represent three separate experiments. Promoter activity was assessed by luciferase assays in HUVEC nucleofected with a luciferase-harboring-pSTAT3-promoter construct (**C**, pSTAT3-TA-Luc) and with siRNA. Values are means ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$.

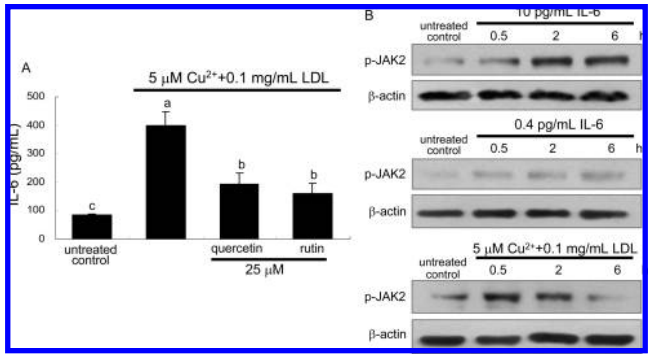

Figure 7. Prevention of IL-6 secretion by quercetin and rutin in Cu^{2+}-oxidized LDL-treated endothelial cells (**A**) and time course activation of JAK2 by IL-6 and Cu^{2+}-oxidized LDL (**B**). HUVEC were pretreated with 25 μM quercetin and rutin in the absence and presence of 5 μM Cu^{2+} and 0.1 mg/mL LDL (**A**). The cell culture medium was collected and measured using ELISA (**A**). Data represent means ± SEM from three independent experiments with multiple estimations. Values not sharing a letter are different at $P < 0.05$. Cells were incubated with IL-6 or with 5 μM Cu^{2+} plus 0.1 mg/mL LDL (**B**). Total cell protein extracts were subjected to Western blot analysis with a primary antibody against phosphorylated JAK2. β-Actin was used as an internal control. Respective bands represent three separate experiments.

(*17*). Accordingly, the pattern of MAPK activation in response to oxidative stress varies in the dependence of the oxidant strength (cell death by apoptosis or necrosis) as well as between cells and even in the same species. Moreover, oxidized LDL promoted the activation of cytosolic tyrosine kinase JAK and STAT through the intracellular oxidative stress by means of its lipid peroxidation products (*17*). The STAT-dependent mechanisms are involved in the transduction of death/survival signals (*18*). It has been also demonstrated that the JAK pathway linked to STAT signaling plays a role in myocardial injury including myocardial infarction and oxidative damage (*27*). This study revealed that Cu^{2+}-oxidized LDL resulted in oxidative stress leading to endothelial apoptosis through enhancing MAPK and JAK signaling.

The antioxidant NAC dampened the activation of JNK and p38 MAPK and the activity of redox-sensitive apoptotic genes (*15*). The antioxidants of berberine and kaempferol diminished oxidized LDL-elicited ROS generation and apoptosis through apoptotic signaling pathways (*11*, *12*). It is assumed that a flavonoid displaying a powerful antioxidant capacity blocks redox-sensitive signaling pathways triggered by various oxidative stimuli including oxidized LDL. Cellular and molecular antiapoptotic features of antioxidants, (−)-epigallocatechin gallate (EGCG) and quercetin, were inferred from their antioxidant effects via both cytosolic and mitochondrial mechanisms (*22*). This study showed that the flavonols of quercetin and rutin diminished Cu^{2+}-oxidized LDL-induced ROS generation and mitigated endothelial apoptosis by modulating cellular apoptotic machinery via initiation of JAK-STAT signaling, leading to differential activation of MAPK-dependent mechanisms. Quercetin appeared to prevent endothelial cell death through other mechanisms independent of JNK signaling despite an involvement of the JAK2-STAT3 pathway. In contrast, rutin exhibited protection against oxidized LDL through hampering MAPK-dependent pathways involving the activation of JAK2. Together, there was a notable linkage of JAK2-STAT3 activation to MAPK signaling in oxidized LDL-induced endothelial apop-

tosis. The difference in the mechanistic signals of quercetin and rutin blocking oxidized LDL-induced endothelial apoptosis cannot be determined from these data. Our previous study revealed that hesperetin, unlike EGCG, hampered the p38MAPK pathway in the interplay with JAK2-MAPK pathways evoked by oxidized LDL (*7*). Differences in the inhibitory mechanisms exerted by individual flavonoids appear to stem from their structures. In addition, the presence of the disaccharide rutinose moiety of quercetin may display differential effects on signaling pathways. Accordingly, it is thought to be worthwhile to investigate another quercetin glycoside, quercitrin, with rhamnose. However, mechanistic evidence data about the specific dietary constituents conferring cytoprotection are still sparse.

Cytokine IL-6 plays an important role in triggering the acute phase response to injury or inflammation (*28*). Both JAK-STAT and MAPK pathways are known to play a major role in IL-6 receptor signaling, leading to activation of downstream signal transduction cascades (*28*). In addition, many investigations have closely examined simultaneous signaling of IL-6 through both JAK-STAT and MAPK pathways (*13*). This study proved that oxidized LDL directly triggered the JAK2-STAT3 pathway independent of the IL-6 receptor signaling. On the other hand, the SOCS3 expression enhanced by rutin presented additional diversity in the JAK-STAT pathway by blunting activation of STAT3 that affects activation of downstream genes. This study did not investigate the interactions between domains containing tyrosine phosphatase 2 (SHP2) and SOCS3 manipulating the JAK2-STAT3 and the MAPK signaling pathways (SHP-2 with MAPK and SOCS3 with JAK/STAT). Nevertheless, the results suggest that boosted SOCS3 may influence signaling via the JAK-STAT and the MAPK pathways. However, the signaling interplay between JAK-STAT and MAPK pathways and the signaling inhibition of SOCS3 influenced by oxidized LDL are still poorly understood. Moreover, therapeutic actions against the JAK-STAT signaling pathway toward resistance to endothelial apoptosis are barely defined.

STAT3 has been shown to be an attractive molecular target for the development of novel cancer therapeutics (*29*). Plant polyphenols such as silibinin, resveratrol, and curcumin effectively interrupted the STAT3 signaling and might have potential significance in therapeutic interventions of the deadly malignancy (*16*, *29*). Luteolin targeted STAT3 through the ubiquitin-dependent degradation in tyrosine 705-phosphorylated STAT3 and the gradual down-regulation in serine 727-phosphorylated STAT3 accompanying inactivation of CDK5, thereby triggering apoptosis via up-regulation in Fas/CD95 (*30*). In addition, there is emerging evidence that disruption of SOCS expression or activity is associated with several immune and inflammatory diseases and that manipulation of SOCS activity may provide a novel therapeutic strategy toward immunological disorders (*31*). In this study SOCS3 was de novo synthesized in response to rutin that hampered the JAK2-STAT3 pathway leading to endothelial apoptosis.

In summary, this study elucidated how the JAK-STAT pathway orchestrates oxidized LDL-elicited cellular damage of the vascular endothelium, along with the potential challenges of quercetin and rutin in manipulating the pathway for cardiovascular therapies. Quercetin and rutin blunted Cu^{2+}-oxidized LDL-triggered oxidative stress and consequent endothelial apoptosis by modulating cellular apoptotic machinery via initiating the JAK-STAT signaling. However, rutin, unlike quercetin, abolished HUVEC apoptosis mediated by MAPK-dependent mechanisms closely linked to JAK2 activation. In addition, oxidized LDL activated endothelial apoptosis through the direct JAK2 signaling but not through IL-6-mediated stimulation. Accordingly, quercetin and rutin appear to switch off apoptotic machinery and hence dampen activation of caspase-3 against the apoptotic triggers. Therefore, dietary interventions with antioxidant compounds such as these flavonoids may provide resistance to cellular oxidative damage and limit atherosclerosis.

ABBREVIATIONS USED

ASK1, activation of apoptosis signal-regulating kinase 1; DCFH, 2′,7′-dichlorodihydrofluorecein diacetate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ERK1/2, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; IL, interleukin; LDL, low-density lipoprotein; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MTT, 3-(4,5-dimetylthiazolyl)diphenyl tetrazolium bromide; pNA, *p*-nitroanaline; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOCS3, suppressor of cytokine signaling 3; STAT, signal transducers and activators of transcription; TBARS, thiobarbituric acid reactive substances; TBS-T, Tris-buffered saline−Tween 20.

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