Different short- and long-term effects of resveratrol on nuclear factor-κB phosphorylation and nuclear appearance in human endothelial cells[1,2]

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ABSTRACT

Background: Resveratrol (a naturally occurring phytoalexin found in grapes and wine) has cardiovascular protective effects that suggest the antiatherogenic (ie, antiinflammatory) activities of the compound on endothelial cells.

Objective: The antiinflammatory activity of resveratrol could be mediated by its interference with nuclear factor-κB (NF-κB)–dependent transcription. Thus, we studied the in vitro influence of physiologic concentrations of resveratrol (≤ 1 μmol/L) on the NF-κB signaling pathway after tumor necrosis factor α (TNF-α) stimulation of endothelial cells.

Design: The effects of a 30-min (acute) and an overnight incubation of resveratrol on the nuclear appearance of p50-NF-κB and p65-NF-κB on serine and tyrosine phosphorylation of the inhibitory subunit κB α (IκBα), cytoplasmic concentrations of IκBα, NF-κB phosphorylation or nitrosylation, the reduction of the mitotic inhibitor p21, and the activation of peroxisome proliferator–activated receptor α were evaluated.

Results: The nuclear appearance of p50-NFκB and p65-NFκB acutely induced by TNF-α was not modified by resveratrol but was increased after overnight incubation with resveratrol alone or in combination with TNF-α. Acute treatment with resveratrol did not modify TNF-α–induced cytoplasmic IκBα serine phosphorylation but did increase IκBα tyrosine phyophorylation. Resveratrol increased the tyrosine phosphorylation (but not nitrosylation) of immunoprecipitated NF-κB, did not decrease cellular p21, and did not increase peroxisome proliferator–activated receptor α activity.

Conclusions: Acute resveratrol treatment does not inhibit the nuclear appearance of NF-κB in human umbilical vein endothelial cells, but overnight treatment does. The increase in tyrosine phosphorylation of IκBα, p50-NF-κB, and p65-NF-κB suggests the involvement of such alterations in the modulation of NF-κB transcription activity. *Am J Clin Nutr* 2003;77:1220–8.

KEY WORDS Resveratrol, endothelial cells, nuclear factor-κB, atherosclerosis, inflammatory disease, red wine, tumor necrosis factor α

INTRODUCTION

Atherosclerosis is an inflammatory disease (1). On the basis of the findings of many pathophysiologic studies in humans and animals, the first step in atherogenesis is endothelial dysfunction (2). The injury to endothelium that leads to endothelial dysfunction is provoked by some possible causes: elevated or modified LDL, hypertension, diabetes mellitus, elevated plasma homocysteine concentrations, infectious microorganisms, and free radicals. This endothelial dysfunction determines compensatory responses that alter the normal homeostatic properties of the endothelium. The different forms of injury increase the adhesiveness of endothelial cells with respect to leukocytes and platelets and remove the anticoagulant functions and the maintenance of the vascular tone by the endothelium (1, 3). Nuclear factor κ-B (NF-κB) is an inducible regulatory system involved in endothelial activation (4, 5). The control of processes leading to endothelial dysfunction could represent a useful tool for reducing the incidence of atherosclerotic lesions, which are responsible for cardiovascular diseases.

In many epidemiologic studies, moderate red wine consumption has been found to exert a cardioprotective effect (6, 7). Resveratrol is a molecule in wine that is thought to have cardioprotective activity (8). Some previous data have indicated that resveratrol has antiinflammatory and anticancer properties (9–12). A possible antiinflammatory role of resveratrol could be related to an interference with the NF-κB signaling pathway, which regulates the expression of various genes involved in inflammation (13, 14). Among the cytokines modulating endothelial functions, the proinflammatory tumor necrosis factor α (TNF-α) plays a crucial activating role (15). After engagement with its receptor, TNF-α regulates the expression of some adhesion molecules and cytokines on endothelial cells and also increases vascular permeability (16–18). After TNF-α stimulation, p50-NF-κB and p65-NF-κB are released by their cytoplasmic precursor and translocate into the nucleus, where they regulate the transcription of some genes (5).

The purpose of the present study was to investigate the antiinflammatory role of resveratrol at the level of endothelial cells. Thus, we studied the mechanism by which resveratrol interferes

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with the NF-κB signaling pathway in TNF-α–stimulated human endothelial cells at concentrations found in vivo in plasma after moderate wine consumption (100 nmol/L or 1 μmol/L) (19). However, taking into account the cardiovascular protective effects of resveratrol in vivo after brief and prolonged consumption of red wine, we studied the in vitro activity of resveratrol on endothelial cells after a brief (30 min, acute) or long (overnight) incubation.

MATERIALS AND METHODS

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated according to established procedures (20); cultured under standard conditions in medium M-199 containing 15% fetal calf serum (Sigma-Aldrich, Milan, Italy), heparin (15 U/mL), and endothelial cell growth factor (20 μg/mL; Boehringer Mannheim, Mannheim, Germany); and used within the 5th passage.

The monolayer of confluent cells was stimulated by the addition of TNF-α (100 U/mL) for 30 min or for overnight incubation. When used, resveratrol (Pharma Science, Montreal) was added to the cells 30 min before the addition of the other agonists. It was dissolved as previously reported (9) and used at the concentrations of 100 nmol/L and 1 μmol/L.

Cytosolic and nuclear extracts

Cytosolic or nuclear extracts were obtained as described (21). Briefly, cells were lysed in a buffer containing 50 mmol tris/L, 10 mmol NaCl/L, 1 mmol EDTA/L, 1 mmol dithiothreitol/L, 1 μmol leupeptin/L, 1 μmol pepstatin/L, 1 μmol aprotinin/L, 1 μmol phenylmethylsulfonylfluoride/L, and 0.5% Nonidet P-40. Cell lysates were centrifuged at 18 000 × *g* for 10 min at 4 °C. The cytosolic fractions (supernatant fluid) were separated, and the nuclei were then extracted from the pellets at 4 °C in the same aforementioned buffer containing 0.4 mol NaCl/ L. The nuclear protein content was measured with the colorimetric method described by Bradford (22) with the use of a kit manufactured by Bio-Rad Laboratories GmbH (Munich, Germany).

For the analysis of inhibitory subunit κB (IκBα), phosphoserine-IκBα, and p21, the samples were lysed directly with Laemly buffer (0.5 mol tris-HCl/L, pH 6.8; glycerol, 10% sodium dodecyl sulfate, β-mercaptoethanol, and 0.05% bromophenol blue).

Immunoprecipitation

Nuclear protein extracts or cytosolic extracts (300 μg) were respectively incubated at 4 °C for 2 h with anti-p50- NF-κB or p65-NF-κB (2 μg; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-IκBα (Santa Cruz Biotechnology) or with control mouse immunoglobulin G. Antibody-protein complexes were recovered by incubation for 1 h with protein G–sepharose (25 μL) and low-speed centrifugation (18 000 × *g*, 15 s, 4 °C). Sedimented materials (the pellets) were washed 3 times with nuclear lysis buffer, resuspended in reducing Laemly buffer, boiled for 5 min, and then immunoblotted.

Immunoblotting

Equal amounts of protein per lane were loaded and run by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Samples were then electroblotted onto nitrocellulose filters (Bio-Rad Laboratories), blocked with 10% nonfat dry milk in tris-buffered saline, and incubated overnight at 4 °C with different first antibodies (anti-p50-NF-κB, Santa Cruz Biotechnology; anti-p65-NF-κB, Santa Cruz Biotechnology; anti-phosphoserine 32–IκBα, Calbiochem, La Jolla, CA; anti-IκB, Santa Cruz Biotechnology; anti-phosphotyrosine, Santa Cruz Biotechnology; anti-nitrotyrosine, Cayman, Cabru, Milan, Italy; anti-p21, Santa Cruz Biotechnology; and histone H1, Sigma-Aldrich), followed by horseradish peroxidase (EC 1.11.1.7)–conjugated specific secondary antibody for 2 h at room temperature. The immunoreactive bands were revealed by luminol reaction (enhanced chemiluminescence) with a luminometer (Amersham, Little Chalfont, United Kingdom).

Enzyme-linked immunosorbent assay

Flat-bottom microwell plates were coated with an antibody against IκBα (Santa Cruz Biotechnology) or with unrelated monoclonal antibody [10 μg/mL in Dulbecco's modified Eagle's medium (DMEM; Celbio, Pero, Milan, Italy), pH 9.3] and blocked with 3% bovine serum albumin in DMEM overnight at 4 °C. Cell lysates were added to the coated plates (300 μg/well) for 1 h at 4 °C. After washing, the plates were stained with anti-phosphotyrosine or with anti-IκBα polyclonal antibody (1:1000 dilution) followed by horseradish peroxidase-goat anti-rabbit antiserum (1:100 000 dilution; Sigma-Aldrich). Samples were then developed with diaminobenzidine (Sigma-Aldrich), and optical density was determined at 405 nm.

Transient transfection assays

Mouse aortic endothelial cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated at 37 °C for 16 h before transfection. Cells were transfected in culture medium without serum for 2 h at 37 °C with the use of the cationic lipid RPR 120535B. The transcription protocol (cell density and time course of the experiment) was optimized for the cell line. Similar amounts of DNA were transfected. The plasmids were obtained as previously described (23). The plasmid pSVβGal (Promega, Madison, WI) was used to control transfection efficiency, whereas the plasmid pBluescript (Stratagene, La Jolla, CA) was used as DNA carrier to set the final amount of DNA at 500 ng/well. The plasmids [pG5TkpGL3 at 100 ng/well (reporter), pGal4-hPPAR-αDEF and pGal4-hPPAR-γDEF at 20 ng/well (expression vectors), and pRL-CMV at 1 ng/well] were dissolved in serum free DMEM supplemented with 150 mmol NaCl/L, 50 mmol sodium bicarbonate/L, and the cationic lipid (6 nmol/μg DNA); vortexed; incubated for 30 min at room temperature; and added to the cells. After 2 h, cells were washed with 0.15 mol NaCl/L, 0.01 mol sodium phosphate buffer/L (pH 7.2), and incubated for 36 h in fresh medium containing 0.2% fetal calf serum and the compounds tested or the vehicle (dimethylsulfoxide, 0.1% by vol). At the end of the experiment, the cells were washed once with ice-cold 0.15 mol NaCl/L and 0.01 mol sodium phosphate buffer/L (pH 7.2), and luciferase (EC 1.13.12.7) activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. All transfection experiments were performed ≥ 3 times, and each transfection was performed in triplicate. The protein content of the extract was evaluated with the Bradford assay by using a kit from Bio-Rad Laboratories (22).

Statistical analysis

The Wilcoxon test for dependent data was used. Significance was assumed at $P < 0.05$. UNISTAT 4.53 (Unistat Ltd, London) was used for the analyses.

RESULTS

Effect of resveratrol on the nuclear appearance of p50-NF-κB and p65-NF-κB in TNF-α–stimulated HUVECs

TNF-α induces the production and expression of some inflammatory and procoagulant molecules in HUVECs (16). These effects are controlled by the NF-κB–signaling pathway. We analyzed the ability of resveratrol to interfere with this pathway.

The time course of p50-NF-κB and p65-NF-κB subunits in cytoplasmic and nuclear fractions of HUVECs after TNF-α stimulation (time = 0) is shown in **Figure 1**. We found that cytoplasmic fractions were not modified with time. The nuclear appearance of p50-NF-κB and p65-NF-κB was induced within 15 min, peaked at 30 min ($P < 0.05$), and was also detectable at 60 min.

The effect of resveratrol (used at concentrations of 100 nmol/L and 1 μmol/L) on nuclear appearance is shown in **Figure 2**. After 30 min of endothelial stimulation with TNF-α, resveratrol alone or associated with TNF-α did not modify the appearance of nuclear p50-NF-κB or p65-NF-κB (Figure 2A). When nuclear appearances were analyzed after overnight incubation with TNF-α, in the presence or absence of resveratrol, the following results were obtained: *1*) TNF-α alone did not induce the appearance of p50-NF-κB or p65-NF-κB and *2*) resveratrol alone or associated with TNF-α induced an increased nuclear appearance of both NF-κB proteins (Figure 2B; $P < 0.05$).

Effect of resveratrol on IκBα activity

NF-κB nuclear appearance is finely regulated by amino acid–specific IκBα phosphorylation. Using a specific antibody that recognizes only the serine (serine 32) or the tyrosine phosphorylated form of IκBα, we studied the effect of resveratrol on IkBα phosphorylation. As shown in **Figure 3**, TNF-α stimulation for 30 min induced an increase in IκBα serine phosphorylation. However, resveratrol did not modify resting nor stimulated IkBα serine phosphorylation after a 30-min (Figure 3A) or overnight (Figure 3B) incubation.

Resveratrol induced a significant increase in IκBα tyrosine phosphorylation in the presence and in the absence of TNF-α after 30 min of incubation (Figure 3A). In contrast, after an overnight incubation, resveratrol alone or associated with TNF-α did not modify the tyrosine phosphorylation of IκBα (Figure 3B). After 30 min of incubation, TNF-α reduced total cytoplasmic IκBα concentrations, whereas resveratrol alone (or associated with TNF-α) did not (Figure 3A). After an overnight incubation, resveratrol alone or associated with TNF-α increased total cytoplasmic IκBα concentrations (Figure 3B).

Effect of resveratrol on NF-κB tyrosine phosphorylation or nitrosylation

We examined other nuclear mechanisms able to influence NF-κB–regulated gene transcription, such as NF-κB phosphorylation, NF-κB nitrosylation, the cellular concentration of the mitotic inhibitor p21, and the activation of other transcription factors, ie, peroxisome proliferator–activated receptors α and g (PPAR-α and PPAR-γ). In our experiments, immunoprecipitated nuclear p50-NF-κB (Figure 3C) and p65-NF-κB (Figure 3D) obtained from HUVECs showed an increase in tyrosine phosphorylation after resveratrol treatment alone and also after treatment with both TNF-α and resveratrol. On the contrary, tyrosine nitrosylation concentrations were not modified by resveratrol treatment.

Effect of resveratrol on endothelial p21 concentrations

The cellular concentration of p21, which binds with p300 cyclin-dependent kinase (EC 2.7.1.37), can regulate NF-κB transcriptional activity (24). In our study, resveratrol alone or associated with TNF-α did not cause a reduction in the cellular concentration of p21 (Figure 3E).

Effect of resveratrol on PPAR endothelial activation

PPARs are involved in the regulation of NF-κB transcriptional activity. In fact, PPAR activators interfere negatively with the NF-κB signaling pathways (25–27). In addition, the effects of resveratrol are comparable with those related to PPAR activation (28–30); for this reason we studied PPAR endothelial activation in the presence of resveratrol.

To test the ability of resveratrol to modulate the activities of PPARs, mouse aortic endothelial cells were transiently transfected with the responsive sequence of PPAR-α or PPAR-γ. The following concentrations of resveratrol were used: 100 nmol/L, 1 μmol/L, 10 μmol/L, and 50 μmol/L. Resveratrol activated PPAR-α, although not significantly, only when used at the concentration of 50 μmol/L; at lower concentrations it did not exert any effect. Resveratrol was also unable to activate PPAR-γ at all of the concentrations studied (**Figure 4**).

DISCUSSION

Resveratrol has been shown to interfere with cytokines (eg, TNF-α and interleukin 1β) or lipopolysaccharide activity in the expression of some genes whose proteins are involved in atherosclerotic processes (10, 11). Resveratrol has been shown to interfere with LDL oxidation (31–33) and to increase nitric-oxide synthase (EC 1.14.13.39) in cultured arterial endothelial cells (34). We previously showed in vitro that resveratrol, when used at the concentrations obtained in plasma after moderate wine consumption, inhibits the expression of some adhesion molecules in TNF-α or lipopolysaccharide-stimulated endothelial cells and also inhibits granulocyte and monocyte adhesion to endothelium (35, 36).

Because the transcription of genes involved in the aforementioned activities is controlled by transcription factors belonging to the κB family, several studies have been performed to investigate the effects of resveratrol on the NF-κB system. Some authors described a reduced nuclear appearance of NF-κB factors (14), and others showed an interference with their transcription activity (37, 38).

However, previous studies showed a role for resveratrol at concentrations > 10 μmol/L. Because the resveratrol concentration in red wine is ≈10–20 μmol/L, it is not plausible that the effect of resveratrol described at such concentrations would be observed in vivo. We suggest that resveratrol activities should be evaluated at physiologic concentrations, ie, ≤ 1 μmol/L (19). Furthermore, because the effects of resveratrol are observed after prolonged consumption of red wine, a long incubation time should be analyzed together with brief incubations.

The current study was carried out in accord with these assumptions. In our study, we showed that resveratrol did not inhibit the nuclear appearance of p50-NF-κB or p65-NF-κB at concentrations of 100 nmol/L and 1 μmol/L within 60 min of stimulation with TNF-α, in agreement with the findings of other investigators who used higher concentrations (37, 38). In agreement with these data, IκBα serine phosphorylation after TNF-α stimulation was not modified by resveratrol treatment.

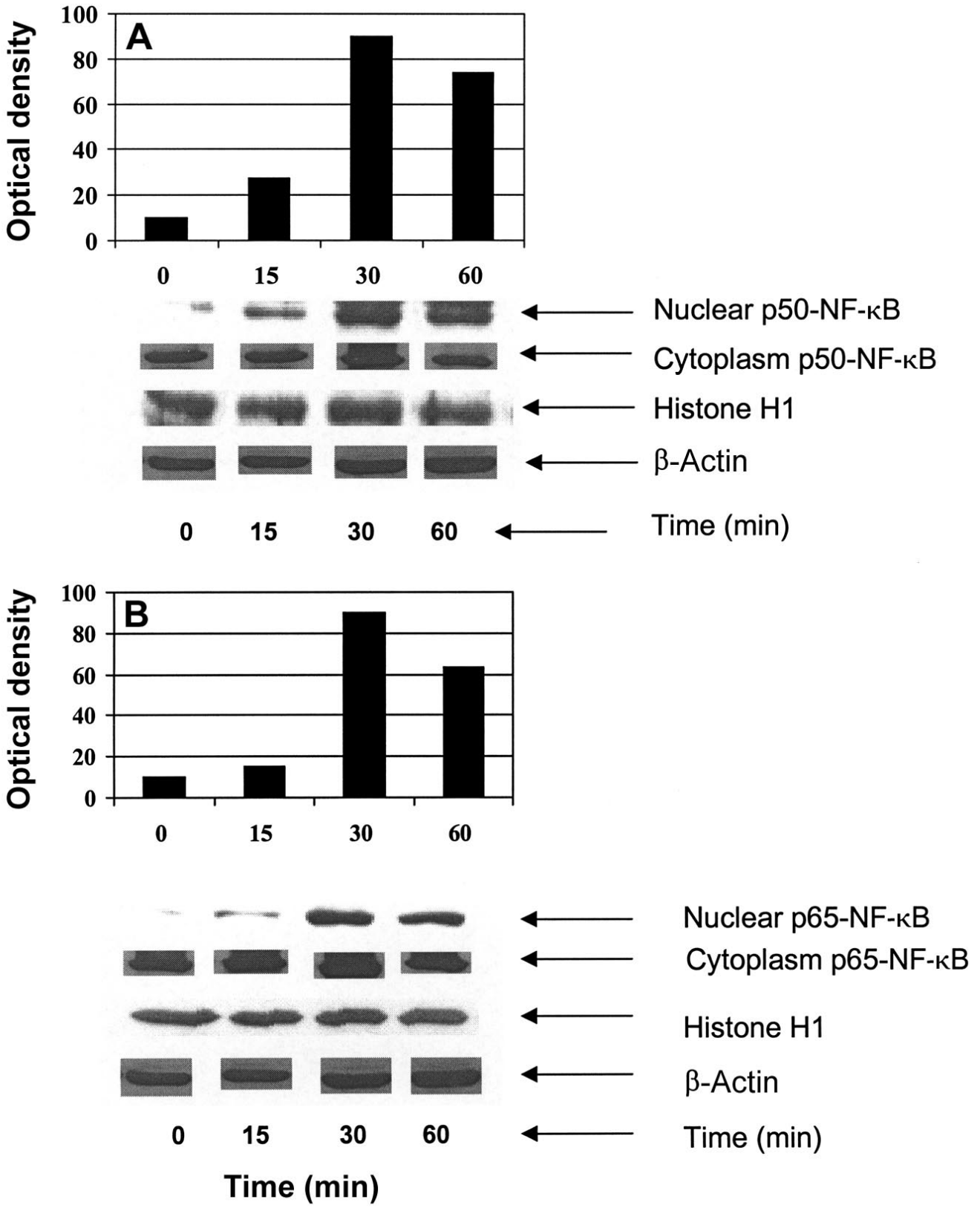

FIGURE 1. Time course of cytoplasmic and nuclear fractions (NFs) of p50-NF-κB (A) and p65-NF-κB (B) after tumor necrosis factor α stimulation (100 U/mL, time = 0). Histone H1 was inserted as a control for nuclear fractions. β-Actin was inserted as a control for cytoplasmic fractions. The optical densities of the nuclear fractions are represented in the histograms. Data represent 1 of 3 similar blots.

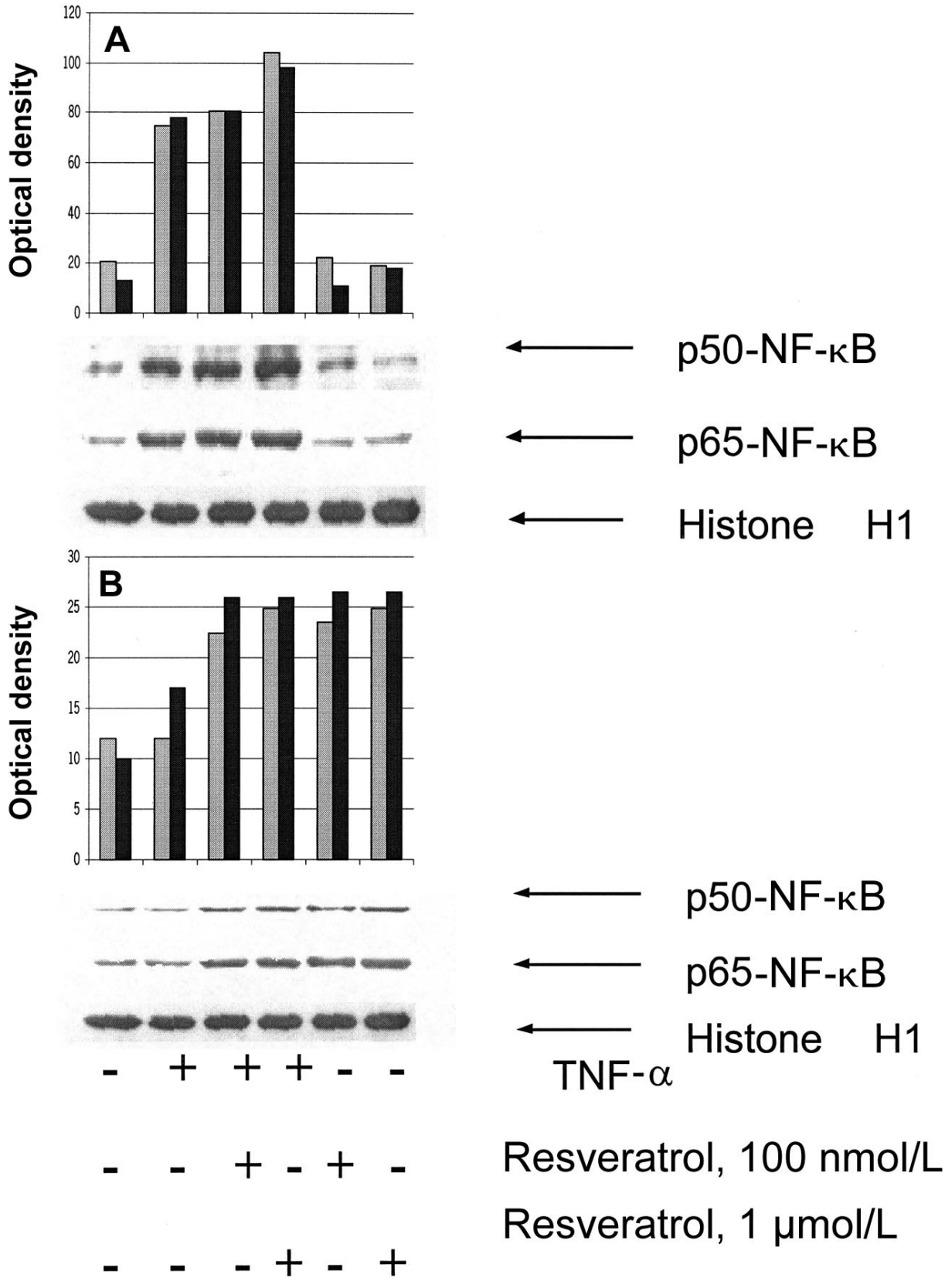

FIGURE 2. Effect of resveratrol alone or associated with tumor necrosis factor α (TNF-α) on the nuclear appearance of the nuclear fractions (NFs) p50-NF-κB (light column) and p65-NF-κB (dark column) after stimulation for 30 min (A) or overnight (B) with TNF-α. Histone H1 was inserted to control the equal protein content of the different lanes. The optical densities of the singular lane are plotted in the histograms. Data represent 1 of 3 similar blots.

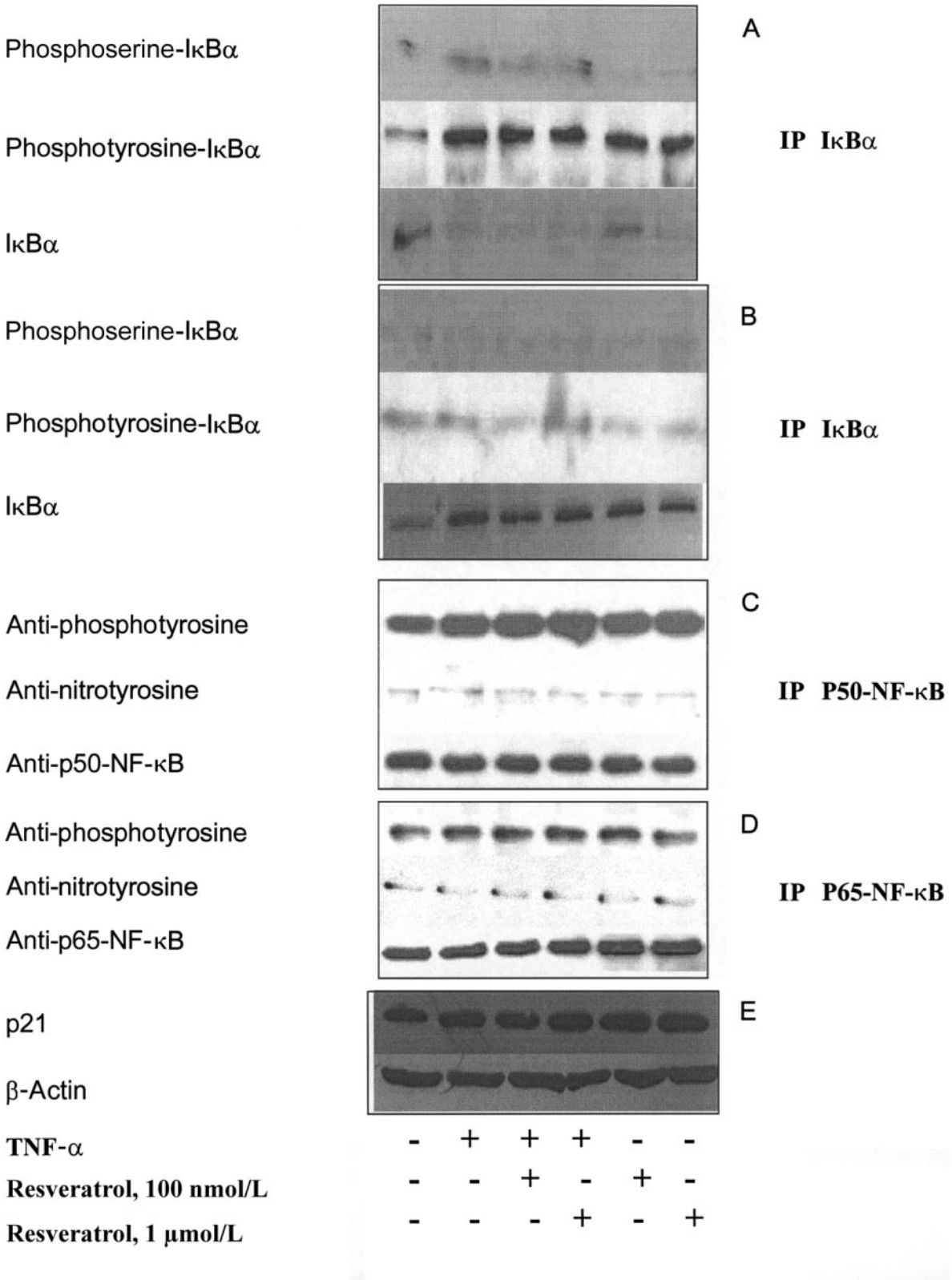

FIGURE 3. Evaluation of phosphoserine-IκBα, phosphotyrosine-IkBα phosphorylation, and total IκBα concentrations of immunoprecipitated (IP) IκBα in cells stimulated for 30 min (A) or overnight (B) with tumor necrosis factor α (TNF-α), as described in Material and Methods. Phosphoserine-IκBα was evaluated by using a specific antibody recognizing only IκBα phosphorylated in serine 32. After IκBα immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, tyrosine phosphorylation was evaluated by anti-phosphotyrosine antibody. Data represent 3 separate experiments. Tyrosine phosphorylation and nitrosylation of IP p50-NF-κB (C) and p65-NF-κB (D) are represented. Cells were stimulated as described in Material and Methods, and tyrosine phosphorylation or nitrosylation was evaluated by Western blot. The figure shows a representative gel. Similar results were obtained in another 3 experiments. P21 and β-actin concentrations measured in total cell lysate are shown in panel E.

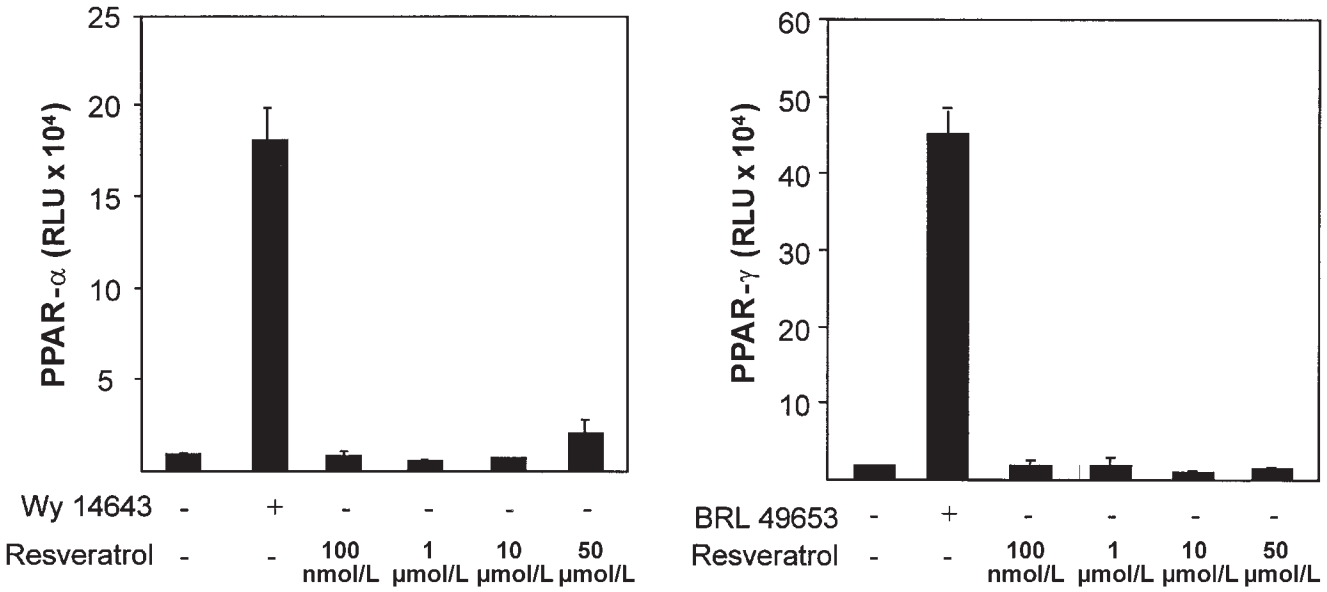

FIGURE 4. Peroxisome proliferator–activated receptor (PPAR) α and γ activation in mouse aortic endothelial cells transiently transfected with the PPAR-α– or PPAR-γ–responsive sequence and exposed to increasing concentrations of resveratrol (100 nmol/L and 1, 10, and 50 μmol/L). PPAR activation was quantified as luciferase activity normalized to the β-galactosidase content. Wy14643 was used as the PPAR-α agonist and BRL49653 as the PPAR-γ agonist. All of the experiments were performed in triplicate. RLU, relative light units.

In contrast with the effect observed after a brief incubation, resveratrol—alone or in combination with TNF-α—induced an increased nuclear appearance of p50-NF-κB and p65-NF-κB when long incubation times were used. Because we showed an increase in the tyrosine phosphorylation of IκBα after 30 min of incubation with resveratrol, we argue that the increased nuclear appearance induced by resveratrol at a longer incubation time reflects a perturbation of the cytoplasmic IκB system. On the other hand, an IκBα-independent pathway of NF-κB activation was described previously (39).

If resveratrol is able to activate the NF-κB signaling pathway, we might suppose that the transcription of genes controlled by these transcription factors is also increased. Consequently, resveratrol should have prothrombotic and proinflammatory actions.

However a protective role of an increase in NF-κB against the damage provoked by ischemia reperfusion has been proposed (40). A beneficial role of resveratrol against the ischemia reperfusion damage has been shown (41). Therefore, we suppose that an increase in NF-κB might be beneficial for the cells. Because different authors have shown a resveratrol-induced impairment of NF-κB transcription (13, 14), we conclude that the increase in NF-κB might be insufficient to induce gene transcription.

In addition, the increase in NF-κB observed after the overnight treatment could reflect an autocrine effect. Such an interpretation could explain the increase in IκBα observed at a longer incubation time. Considering that some studies showed that resveratrol interfered with the transcription ability of NF-κB, we evaluated 4 possible mechanisms by which such transcription might be impaired: p50-NF-κB and p65-NF-κB tyrosine phosphorylation, p50-NF-κB and p65-NF-κB tyrosine nitrosylation, p21 cellular concentrations, and PPAR activation. Our results showed that resveratrol induced a significant increase in p50-NF-κB and p65-NF-κB tyrosine phosphorylation, but it did not significantly modify p50-NF-κB or p65-NF-κB tyrosine nitrosylation, p21 cellular concentrations, or PPAR activation in endothelial cells.

The tyrosine phosphorylation of NF-κB is efficient in modulating transcription activity (4, 5). The physiologic phosphorylation of p50-NF-κB and p65-NF-κB can be observed after TNF-α stimulation and is considered useful for its activation (13). However, protein tyrosine kinase p56lck (EC 2.7.1.112) is not required for TNF-induced activation of NF-κB (42). Furthermore, a phosphatidylinositol 3 kinase (EC 2.7.1.37)/AKT pathway (activated by TNF-α or interleukin 1) does not activate NF-κB in human endothelial cells (43). Such results indicate that some phosphorylations are deleterious for NF-κB transcription activation.

The increase in tyrosine phosphorylation induced by resveratrol on IκBα, p50-NF-κB, and p65-NF-κB indicates that resveratrol possibly acts through a tyrosine kinase activation. Previous studies have indicated an ability of resveratrol to inhibit several protein kinases and in particular some tyrosine kinases (44, 45). Our results do not agree with such studies. However, the fact that resveratrol can inhibit some kinases does not mean that all the kinases are inhibited. In addition, a feature of resveratrol is its dual activity at different concentrations. Indeed, at lower concentrations (< 1 μmol/L) it activates extracellular signal-regulated kinases 1 and 2 in SH-SY5Y cells, whereas at higher concentrations it inhibits such activation (46).

More recent studies have indicated that NF-κB activation requires tyrosine phosphorylation and degradation of IκBα (47). Because we used lower resveratrol concentrations than were used in these other studies, we suppose that an undefined kinase is activated by resveratrol and that such activation is responsible for the cellular perturbations that lead to altered NF-κB transcription activity.

Our data confirm the notion that resveratrol modulates endothelial cell activation in many ways in relation to the way that the

compound is used: at different concentrations and at different incubation times. These results might explain the conflicting in vivo effects on the cardiovascular system that were observed after different quantities and time periods of wine consumption (48–50). The effects of resveratrol are also possibly due to the presence in wine of other products, such as antioxidants and other polyphenols, that are able to down-regulate or up-regulate cardiovascular functions (51). Our data also suggest that the in vivo expression of antiinflammatory and anticoagulant proteins in endothelial cells is influenced by many cooperating factors in blood (eg, TNF and angiotensin) that either induce or inhibit such expression (13, 52).

FP and AF contributed to the study design and to the research and collection of data on the nuclear appearance of p50-NF-κB and p65-NF-κB. AAEB contributed to the study design. BS and CD conducted the experiments and collected data on PPAR-α and PPAR-γ activities. MEF contributed to the study design, evaluated the results, and wrote the manuscript.

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