The NADPH oxidase inhibitor apocynin induces nitric oxide synthesis via oxidative stress

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The NADPH oxidase inhibitor apocynin induces nitric oxide synthesis via oxidative stress.

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ABSTRACT.

We have recently shown that apocynin elicits an oxidative stress in N11 mouse glial cells and other cell types. Here we report that apocynin increased the accumulation of nitrite, the stable derivative of nitric oxide (NO), in the extracellular medium of N11 cell cultures, and the NO synthase (NOS) activity in cell lysates. The increased synthesis of NO was associated with increased expression of inducible NOS (iNOS) mRNA, increased nuclear translocation of the redox-sensitive transcription factor NF-kB and decreased intracellular level of its inhibitor IkBα. These effects, accompanied by increased production of H_2O_2, were very similar to those observed after incubation with bacterial lipopolysaccharide (LPS) and were inhibited by catalase. These results suggest that apocynin, similarly to LPS, induces increased NO synthesis by eliciting a generation of reactive oxygen species (ROS), which in turn causes NF-kB activation and increased expression of iNOS. Therefore, the increased bioavailability of NO reported in the literature after in vivo or in vitro treatments with apocynin might depend, at least partly, on the drug-elicited induction of iNOS, and not only on the inhibition of NADPH oxidase and the subsequent decreased scavenging of NO by oxidase-derived ROS, as it is often supposed.

Keywords: apocynin, glial cells, nitric oxide, nuclear factor-kB, oxidative stress, catalase, lipopolysaccharide, NADPH oxidase.

INTRODUCTION.

The physiopathological importance of NADPH oxidases has led many researchers to use in their experimental studies several inhibitors of these enzymes, mainly diphenyleneiodonium and apocynin (Cai et al., 2003). Apocynin, also named acetovanillone or 1-(4-hydroxy-3-methoxyphenyl)ethanone, is a small molecule extracted from the medicinal herb *Picrorhiza kurroa*, which has been deemed particularly promising as a potent inhibitor of NAD(P)H oxidases. This agent blocks oxidase assembly (Stolk et al., 1994), is effective when administered orally (Beswick et al., 2001), and has been used extensively in several animal models of inflammation, including arthritis, septic shock and asthma (Cai et al., 2003). Several evidences suggest that apocynin is not specific for the NAD(P)H oxidases, because it inhibits thromboxane A_2 formation in guinea pig pulmonary macrophages (Engels et al., 1992) and cytochrome P450 activity in endothelial cells (Pietersma et al., 1998), induces the AP-1 transcription factor in alveolar epithelial cells (Lapperre et al., 1999), interferes with actin polymerization and cytoskeletal rearrangement in polymorphonuclear granulocytes (Muller et al., 1999). We have recently shown that in N11 glial cells apocynin induces an oxidative stress, as suggested by the increase of malonyldialdehyde level (index of lipid peroxidation) and H_2O_2 concentration, by the decrease of the intracellular glutathione/glutathione disulfide ratio and by the activation of pentose phosphate pathway (PPP) (Riganti et al., 2006). These effects, accompanied by an increased release of lactate dehydrogenase in the extracellular medium, have been observed also in other cell types, such as human erythrocytes and epithelial cells, suggesting that apocynin can exert cytotoxic effects through an oxidative stress (Riganti et al., 2006). Thus, some effects of apocynin in in vitro and in vivo experimental models could not depend on NADPH oxidase inhibition, and should be interpreted with caution.

The consequences of the generation of oxidants in cells do not appear to be limited to promotion of deleterious effects. Reactive oxygen species (ROS) may serve also as subcellular messengers in gene regulatory and signal transduction pathways. Many effects of redox changes on cells are mediated either directly or indirectly through the modulation of the nuclear factor-kB (NF-kB) signalling pathway (Allen and Tresini 2000). NF-kB is a dimeric transcription factor that is activated by a variety of cellular stresses (cytokines, bacterial lipopolysaccharide, viral proteins, radiations, oxidative stress, etc.) and is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response, including cytokines, chemokines, lipid mediators, enzymes, adhesion molecules and nitric oxide (NO) (Ali and Mann, 2004).

NO is synthesized by three NO synthase (NOS) isoforms, which favor the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry; in oxygenated living systems, NO is rapidly converted into nitrite and nitrate (Wink and Mitchell, 1998). NO is known to play an important role as a vasodilator and neurotransmitter, but may be also highly cytotoxic: huge amounts of this radical are produced owing to the activation of the inducible NOS (iNOS) isoform in macrophages and other cells, contributing to the inflammatory response (Wink and Mitchell, 1998). The iNOS expression is regulated by various signal transduction pathways, including NF-κB (Korhonen et al., 2005).

In preliminary experiments we have found that apocynin increased the accumulation of nitrite, the stable derivative of NO, in the extracellular medium of N11 cell cultures: aim of this work has been to investigate whether such NO generation was related to the oxidative stress elicited by apocynin and whether it was associated with increased expression of iNOS and activation of the redox-sensitive NF-kB transcription factor.

MATERIALS AND METHODS

Cells and reagents. The N11 mouse glial cell line was a gift from Dr. Marco Righi (CNR Institute of Neuroscience, Section of Cellular and Molecular Pharmacology, Milan, Italy). Cells were cultured up to the confluence in 35- (measurements of nitrite, H_2O_2 and enzyme activities) or 100-mm (other experiments) diameter Petri dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and then incubated for 6 or 24 h in the absence or presence of apocynin, as described in Results. Plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA); electrophoresis reagents were purchased from Biorad Laboratories (Hercules, CA); the protein content of cell monolayers or cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). When not differently indicated, reagents were from Sigma Chemical Co. (St. Louis, MO). Apocynin was dissolved firstly in absolute ethanol and then diluted further in culture medium, in order to obtain in our experimental conditions a final concentration of ethanol not exceeding 0.1% (v/v). Catalase (H_20_2:H_20_2 oxidoreductase, EC 1.11.1.6) from bovine liver was directly solubilized in the culture medium. The final concentration of catalase (1000 U/ml) was chosen after having checked, with preliminary experiments, the optimal concentration of enzyme (from 0.1 to 1000 U/ml) apt to decrease the H_2O_2 generation in cell cultures incubated for 24 h in the absence or presence of 300 μM apocynin (data not shown).

Nitrite production. Confluent cell monolayers in 35-mm diameter Petri dishes were incubated in fresh medium under the experimental conditions indicated in Results. Then nitrite production was measured by adding 0.15 ml of cell culture medium to 0.15 ml of Griess reagent (Ghigo et al., 1998) in a 96-well plate, and, after a 10 min incubation at 37°C in the dark, absorbance was

measured at 540 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). A blank was prepared in the absence of cells and its absorbance was subtracted from that measured in the samples. Sodium nitrite was used as a standard to build the calibration curve. Nitrite concentration was expressed as nmol nitrite/h/mg cell proteins.

Measurement of NOS activity. Cells grown at confluence in 35-mm diameter Petri dishes, after incubation under the experimental conditions described in Results, were detached by trypsin/EDTA, washed with PBS, re-suspended in 0.3 ml of Hepes/EDTA/DTT buffer (20 mM Hepes, 0.5 mM EDTA, 1 mM DTT, pH 7.2) and sonicated on crushed ice with two 10 s bursts. NOS activity was measured on 100 μg of cell lysates with the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research, Oxford, MI). This method employs a NADPH recycling system that permits NOS to catalyze NO production at a constant level for many hours. The stable NO degradation product nitrite accumulated during this period is determined using the Griess reagent. Results were expressed as nmol nitrite/min/mg cell protein.

Measurement of H_2O_2 release. Confluent cell monolayers in 35-mm diameter Petri dishes were incubated for 6 h or 24 h in fresh medium under the experimental conditions indicated in Results. After incubation, cells were washed with fresh medium, detached with trypsin/EDTA, washed with PBS, and then the H_2O_2 concentration was checked with the fluorescent probe Amplex Red, as previously described (Riganti et al., 2006).

Lactate dehydrogenase (LDH) leakage. Confluent cell monolayers cultured in 35-mm diameter Petri dishes were incubated for either 6 or 24 h in fresh medium under the experimental conditions indicated in Results. After incubation, LDH activity was measured on 100 μl of the supernatant and 10 μl of cell lysate, as previously described (Riganti et al., 2006), by recording the oxidation of NADH, detected as decrease of its absorbance at 340 nm with time. Both intracellular and extracellular enzyme activity was expressed as μmol NADH oxidized/min/dish, then extracellular LDH activity was calculated as percentage of the total LDH activity in the dish.

Measurement of pentose phosphate pathway (PPP) activity. Cells were washed with fresh medium, detached with trypsin/EDTA (0.05/0.02% v/v), washed with PBS, and resuspended at 5 x 10^5 cells in 1 ml of Hepes-buffered solution (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes-Na, 10 mM glucose, 1 mM $CaCl_2$, pH 7.4 at 37°C) containing 2 μCi [1-^{14}C]glucose or [6-^{14}C]glucose (Dupont-New England Nuclear, Boston, MA); the metabolic fluxes through the PPP and the tricarboxylic acid cycle were measured after a 6 h incubation at 37°C under the experimental conditions described in Results, by detecting the amount of $^{14}CO_2$ developed from [^{14}C]glucose, as previously described (Riganti et al., 2002; Riganti et al., 2006). The PPP metabolic flux (expressed as nmol CO_2/h/mg cell proteins) was obtained by subtracting the amount of CO_2

developed from [6-^{14}C]glucose from the CO_2 released from [1-^{14}C]glucose (Riganti et al., 2002; Riganti et al., 2006).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was obtained by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). 30 ng of total RNA was reversely transcribed into cDNA with the Superscript™ II One-Step RT-PCR System with Platinum Taq DNA Polymerase (cycling conditions: 1 cycle 50°C for 30 min, 1 cycle 94°C for 2 min). cDNA products were determined by PCR amplification, carried out in a total volume of 50 µl, according to the manufacturer's recommendations. The RT-PCR efficiency was controlled by amplifying a β-actin fragment, used as the product of an housekeeping gene. PCR primers were synthesized by Life Technologies (Milan, Italy). Primers for iNOS (0.25 µM) were: 5'-GGAGATCAATGTGGCTGTGC-3', 5'-AAGGCCAAACACAGCATACC-3' (631 bp); primers for β-actin (0.5 µM) were: 5'-GGTCATCTTCTCGCGGTTGGCCTTGGGGT-3', 5'-CCCCAGGCACCAGGGCGTGAT-3' (230 bp). PCR amplification for iNOS: 1 cycle of denaturation at 95°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 2 min, and 1 cycle of extension at 72°C for 7 min. PCR amplification for β-actin: 1 cycle of denaturation at 95°C for 2 min, 32 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, elongation at 72°C for 1 min, and 1 cycle of extension at 72°C for 7 min. Samples were electrophoresed in 1.5% agarose gels containing ethidium bromide in Tris-acetate/EDTA buffer to visualize the PCR products.

Electrophoretic mobility shift assay (EMSA). Cells were plated in 100-mm diameter dishes at confluence and all procedures for nuclear protein extraction were performed at 4°C using ice-cold reagents, as described (Aldieri et al., 2003). Cells were mechanically scraped in PBS, washed and resuspended (1 x 10^7 cells/0.5 ml) in lysis buffer A [15 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (PMSF), 1 mM DTT, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 0.1% NP-40, pH 7.6]. This suspension was incubated for 10 min on ice with occasional vortexing, and centrifuged for 30 s at 13,000 x g to pellet nuclei, which were rinsed with 0.2 ml of wash buffer B (2 M KCl, 25 mM Hepes, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 µg/ml aprotinin, 2 µg/ml leupeptin, pH 7.6) and incubated at 4°C for 20 min. Then an equal volume of buffer C (25 mM Hepes, 0.1 mM EDTA, 20% glycerol, pH 7.6) was added, the mix was centrifuged at 20,000 x g, and the supernatant stored at -80° until used for EMSA. The probe containing the NF-kB oligonucleotide consensus sequence was labeled with [γ-^{32}P]ATP (Amersham International) (3,000 Ci/mmol, 250 µCi), using T4 polynucleotide kinase (Roche, Basel, Switzerland). The sequence of oligonucleotide was (binding site underlined): 5'-AGTTGAGGGGACTTTCCCAGG-3' (Promega Corporation, Madison, WI). 10 µg of extracts were incubated for 20 min with 20,000 cpm of ^{32}P-labeled double-stranded oligonucleotide at 4°C

in a reaction mixture containing: 2 µl of 10 µg/ml BSA, 2 µl of buffer D (100 mM KCl, 20 mM Hepes, 0.5 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 20% glycerol, 0.25% NP-40, pH 7.6), 4 µl of buffer E (300 mM KCl, 100 mM Hepes, 10 mM DTT, 100 µM PMSF, 20% Ficoll, pH 7.6) and 2 µg of poly(dI-dC) (Roche). The final volume of the mix was brought to 25 µl with water. The DNA-protein complex was separated on a not-denaturating 4% polyacrilamide gel in TBE buffer (0.4 M Tris, 0.45 M boric acid, 0.5 M EDTA, pH 8.0). After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 48 h.

Western blot analysis. Cells were directly solubilized in the lysis buffer (25 mM Hepes, 135 mM NaCl, 1% NP40, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol), supplemented with protease inhibitor cocktail set III [100 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 80 µM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin; Calbiochem, La Jolla, CA], 2 mM PMSF and 1 mM sodium orthovanadate. Whole cell extracts containing 15 µg of proteins were separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with an anti-IkBα antibody (from rabbit, diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology, Santa Cruz, CA). After a 1 h incubation, the membrane was washed with PBS-Tween 0.1% and subjected for 1 h to a peroxidase-conjugated anti-rabbit antibody (Biorad, diluted 1:3000 in PBS-Tween with Blocker Not-Fat Dry Milk 5%, Biorad). The membrane was washed again with PBS-Tween and proteins were detected by enhanced chemiluminescence (Immun-Star, Biorad).

Statistical analysis. All data in text and figures are provided as means $\pm$ SEM. The results were analysed by a one-way Analysis of Variance (ANOVA) and Tukey's test: the data reported in the Fig. 1, showing the effects of varying concentrations and incubation time periods with apocynin, have been also analysed by a two-way ANOVA with Dunnett's test . $p < 0.05$ was considered significant.

RESULTS

Apocynin stimulates nitrite production and NOS activity in N11 cells. The incubation with apocynin increased the nitrite production and the NOS activity of N11 cells: this effect was evident starting from 300 µM apocynin (6 h incubation) and from 30 µM apocynin (24 h incubation) (Fig. 1). At the highest concentrations, the effect of apocynin was similar to that elicited by bacterial lipopolysaccharide from *Escherichia coli* (LPS), a well-known iNOS inducer (Fig. 1). A similar incubation with apocynin and LPS for 1 h and 3 h did not elicit changes of nitrite levels and NOS activity (data not shown). The 300 µM concentration of apocynin was chosen to perform subsequent experiments: such a concentration is equal to or lower than that used in other

experimental works (Yoshida et al., 2001; Schimmel and Bauer, 2002; McNally et al., 2003; Rupin et al., 2004; Ali et al., 2004; Riganti et al., 2006).

ROS scavenging inhibits the production of nitrite and the cytotoxic effect induced by apocynin in N11 cells. Apocynin and LPS induced in N11 cells an increased generation of H_2O_2 after 1 h of incubation, that persisted after longer incubation times (Fig. 2). The coincubation with the antioxidant enzyme catalase markedly inhibited the level of H_2O_2 (Fig. 2). The increase of NO synthesis induced in N11 cells by apocynin and LPS was associated also with augmented release of LDH into the extracellular medium (Fig. 3): catalase inhibited both the production of nitrite and the release of LDH observed after a 6 or 24 h incubation with apocynin or LPS (Fig. 3). ROS generation is generally followed by an increase of the activity of PPP, the main antioxidant pathway in eukaryotic cells: the oxidative stress, consuming NADPH, activates allosterically the pacemaker enzyme of PPP, i.e. the glucose 6-phosphate dehydrogenase, thus allowing to restore NADPH and glutathione (Eggleston and Krebs, 1974). Both apocynin and LPS elicited the activation of the PPP, which was blunted when they were incubated with the cells in the presence of catalase (Fig. 4).

The cytotoxic effect of apocynin and LPS on N11 cells is NO-mediated. In the presence of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), neither LPS nor apocynin was able to increase the nitrite levels and the LDH release in the culture medium after 6 or 24 h (Fig. 5). Since in the same experimental conditions L-NMMA did not modify the production of H_2O_2, NO seems to be the main effector of the cytotoxic effect of apocynin and LPS (Fig. 5).

Apocynin increases the expression of iNOS mRNA via an oxidative stress. In RT-PCR experiments we observed that a 6 h and 24 h incubation with apocynin, as well as with LPS, increased the expression of iNOS mRNA in N11 cells (Fig. 6). After shorter times (1 h and 3 h) of incubation no modification was detectable (data not shown). The iNOS induction elicited by apocynin and LPS was completely reverted by catalase, thus confirming the central role of the oxidative stress in the iNOS induction observed in N11 cells incubated with apocynin (Fig. 6).

Apocynin elicits the nuclear translocation of the redox-sensitive transcription factor NF-kB, which is inhibited by ROS scavenging. In unstimulated N11 cells, NF-kB was nearly absent in the nucleus (Fig. 7, A), while its cytosolic inhibitor IkBα was abundant (Fig. 7, B). On the contrary, a 6 h incubation with apocynin clearly promoted the NF-kB nuclear translocation, an index of its activation, and markedly reduced the cytosolic level of IkBα, an index of its degradation. LPS exerted the same effect (Fig. 7, A and B). Catalase completely prevented the apocynin- and LPS-induced nuclear translocation of NF-kB and decrease of cytosolic IkBα (Fig. 7). These changes were detectable also after a longer (24 h) but not after shorter (1 and 3 h) incubation times (data not shown).

The inhibition of the nuclear translocation of NF-kB prevents the increase of NO synthesis and the cytotoxic effect elicited by apocynin. To clarify the role of NF-kB in inducing NOS activity, we performed the same experiments in the presence of the SN50 (Calbiochem, La Jolla, CA), a compound which blocks the nuclear localization of NF-kB: after a 6 h incubation SN50 inhibited, as expected, the apocynin- and LPS-induced nuclear translocation of NF-kB and in parallel prevented the decrease of IkBα (Fig. 7). Under the same experimental conditions, SN50 completely prevented the apocynin- and LPS-induced increase of nitrite accumulation, NOS activity and LDH release (Fig. 8).

DISCUSSION

We have recently shown apocynin to induce an oxidative stress and exert a cytotoxic effect in N11 mouse glial cells and in human epithelial cells (Riganti et al., 2006). Subsequently, we found that apocynin induces also the accumulation of nitrite, the stable derivative of NO, in the extracellular medium: thus, we performed experiments whose results suggested that the generation of ROS is required for apocynin to stimulate the synthesis of NO in N11 cells.

Various signal transduction pathways have been suggested to regulate the iNOS expression (Korhonen et al., 2005). Since the apocynin-induced NO synthesis appeared to be dependent on the generation of an oxidative stress, we have studied the redox-sensitive transcription factor NF-kB, a term which includes a family of dimeric transcription factors controlling a large number of genes in response to various cellular stresses (Viatour et al., 2005). Indeed, binding sites for the transcription factors NF-kB have been found in the promoter region of both mouse and human iNOS gene (Korhonen et al., 2005).

As a positive control of NF-kB-mediated induction of iNOS we have chosen LPS (endotoxin), the main component of the cell wall of Gram-negative bacteria and a well-known inducer of genes involved in the expression of inflammatory mediators (Macdonald et al., 2003). During sepsis, LPS activates in monocytes and neutrophils a number of intracellular signalling pathways, including the IkB kinase α (IKKα). In resting cells, the nuclear localization of NF-kB is hindered by the binding of the inhibitory protein IkBα, which sequesters NF-kB in the cytoplasm. When activated, IKKα phosphorylates IkBα, leading to its ubiquitinylation and degradation and allowing NF-kB translocation to the nucleus (Viatour et al., 2005).

N11 glial cells are macrophage-like mouse cells (Righi et al., 1989) very sensitive to LPS as far as the stimulation of NO synthesis is concerned (Aldieri et al., 2003), and represent a good target model for putative activators of the IKK/NF-kB pathway. Indeed, LPS (20 μg/ml) evoked a clear increase of nitrite accumulation and NOS activity, already detectable after 6 h of incubation. Apocynin elicited the extracellular accumulation of nitrite, within a range of drug concentrations

commonly used in in vivo and in vitro experiments. In parallel, the NOS activity in the lysates of cells incubated with apocynin for 6-24 h was increased. At the highest doses of apocynin, the increase of NO synthesis and NOS activity was similar to that evoked by LPS.

Both 300 μM apocynin and 20 μg/ml LPS elicited an increased production of H_2O_2, already detectable after only 1 h and persisting after longer incubation times, up to 24 h. The presence in the extracellular medium of catalase, a potent antioxidant enzyme that actively converts H_2O_2 to oxygen and water (Kirkman and Gaetani, 2007), prevented such intracellular accumulation of peroxide. Previous studies have demonstrated that extracellular administration of catalase can inhibit peroxide-sensitive signaling pathways (Nemoto et al., 2000). In some cells, this is because catalase is apparently internalized by an unknown mechanism (Sundaresan et al., 1995; Bae et al., 1999), while in other cells extracellular catalase may act as a sink for the freely diffusible peroxide that is formed intracellularly, promoting diffusion of the peroxide through the membrane, followed by its scavenging (Nemoto et al., 2000; Tommasini et al., 2002).

The capacity of both apocynin and LPS to stimulate ROS generation was confirmed by their ability to elicit the activation of PPP, which was blunted in the presence of catalase. LPS is known for a long time to induce oxidative stress in many cell types: it is supposed to stimulate ROS generation via activation of NADPH oxidase (Chiang et al., 2006), and according to this model PPP is necessary in providing NADPH to maintain this reaction. The mechanism by which LPS activates NADPH oxidase is still under investigation (Iles and Forman, 2002; Park et al., 2004).

As to apocynin, we have recently observed (Riganti et al., 2006) that in N11 cells it causes an oxidative stress (probably mediated by a redox cycle triggered at its phenolic ring by metal ions such as copper), which is associated with lipoperoxidation and depletion of GSH: these events are accompanied by the activation of PPP, which is prevented when the intracellular GSH pool of N11 cells is restored (by incubating the cells with apocynin together with GSH).

Since catalase, acting as a ROS scavenger, inhibited the activation of PPP induced by both apocynin and LPS, the traditional hypothesis suggesting that PPP activation is subsequent to the LPS-induced activation of NADPH oxidase should be investigated more thoroughly. Indeed, LPS could exert the oxidative stress with other mechanisms. The hypothesis that LPS increases the ROS production via NADPH oxidase is mainly based on the evidence that diphenyleneiodonium and apocynin prevent the LPS-elicited ROS generation. But we have previously provided evidences that both compounds (Riganti et al., 2004; Riganti et al., 2006) are not specific inhibitors of NADPH oxidase and may exert other effects (Emre et al., 2007).

Apocynin- and LPS-induced nitrite accumulation was completely inhibited by catalase, confirming that ROS generation is an important intermediate of LPS-triggered intracellular signalling pathway (Chiang et al., 2006) and showing for the first time that the oxidative stress is

necessary also for apocynin to stimulate NO synthesis. Catalase inhibited also the LDH leakage caused by apocynin and LPS, suggesting that the oxidative stress was responsible of their cytotoxicity.

Since NO itself may be cytotoxic in many cellular models (Wink and Mitchell, 1998), we assessed whether the apocynin-elicited NO synthesis and LDH release were related phenomena. In our experimental conditions the NOS inhibitor L-NMMA efficiently inhibited the accumulation of nitrite and the release of LDH, but it did not modify the H_2O_2 generation induced by apocynin and LPS. This suggests that NO is a major responsible for the cytotoxicity of apocynin and LPS in our experimental model, although we do not exclude that NO could exert its toxic effect also by reacting with ROS and generating peroxynitrite; catalase could prevent also this reaction.

The increased synthesis of NO in N11 cells incubated with apocynin or LPS was associated with increased expression of iNOS mRNA, increased translocation of NF-kB to the nucleus and decreased intracellular level of IkBα. While the increased generation of H_2O_2 was already detectable after a 1 h incubation with apocynin and LPS, each of these effects was detectable after a 6 h incubation and was clearly inhibited when catalase was present in the culture medium: this suggests that the oxidative stress precedes the activation of NF-kB and NOS. The results we have obtained in N11 cells incubated with LPS are in line with previous data concerning the ability of antioxidants to inhibit NF-kB activation in LPS-treated murine macrophages (Kelly et al., 1994). The NF-kB inhibitor SN50 prevented the apocynin- and LPS-induced nuclear translocation of NF-kB and decrease of IkBα, as well as the apocynin- and LPS-induced increase of nitrite accumulation, NOS activity and LDH release.

Taken as a whole, these results suggest that apocynin, similarly to LPS, induces increased NO synthesis by eliciting a generation of ROS, which in turn activates NF-kB and elicits increased expression of iNOS. It has been reported that apocynin reduces the NF-kB activation in H9c2 rat cardiac cells and in C2C12 mouse myoblasts (Piao et al, 2005), and in rat aortic smooth muscle cells (Zhou et al, 1999): the different experimental conditions may account for this discrepancy with our results, since in those experiments apocynin was incubated with the cells for longer time periods (6 days) (Piao et al, 2005) or at higher concentrations (2-5 mM) (Zhou et al, 1999).

Previously, apocynin has been shown to increase NO levels in different biological systems: this observation has been interpreted as a consequence of the inhibition of NADPH oxidase, causing a decreased generation of superoxide, which is a potential NO scavenger. Apocynin has been found to cause relaxation of arteries in many in vivo experiments (see for instance: Hamilton et al., 2002; Lopez et al., 2003; Hayashi et al., 2005; Seto et al., 2006; Matsumoto et al, 2007), suggesting that the NO/superoxide balance is a key regulator of endothelial function. This result has been used as an evidence of the role of NADPH oxidase-derived superoxide in lowering NO effects in diabetes,

aging, hypertension, etc., and has raised interest about the possibility to use apocynin or other oxidase inhibitors in the treatment of cardiovascular pathologies. The results of the present work suggest that apocynin may favor NO synthesis without involving the NADPH oxidase system.

Recently, the improvement of vascular relaxation and endothelial functions elicited by apocynin has been related to the increase of neuronal (Paliege et al, 2006) or endothelial NOS (Fukatsu et al, 2007) activity. However, both endothelial and smooth muscle cells may express iNOS activity (Binion et al., 1998; Lee et al., 2004; Ozawa et al., 2004; Hecker et al., 1999) and are likely to produce NO as a consequence of apocynin-induced NF-kB stimulation. The induction of NF-kB translocation and NO synthesis elicited by apocynin is not cell- or species-dependent: indeed, in the MH-S murine alveolar macrophages and in the A549 human lung epithelial cells we obtained results superimposable to those observed in the N11 cells (data not shown). So far, we think that any conclusion concerning apocynin-induced improvement of NO signalling should take into account the possibility that the higher availability of NO might depend on the drug-elicited induction of iNOS, rather than on the reduced scavenging of NO by ROS. Our results pose a further caveat to the use of this NADPH oxidase inhibitor, and should stimulate the use and design of new, more specific inhibitors.

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FIGURE LEGENDS

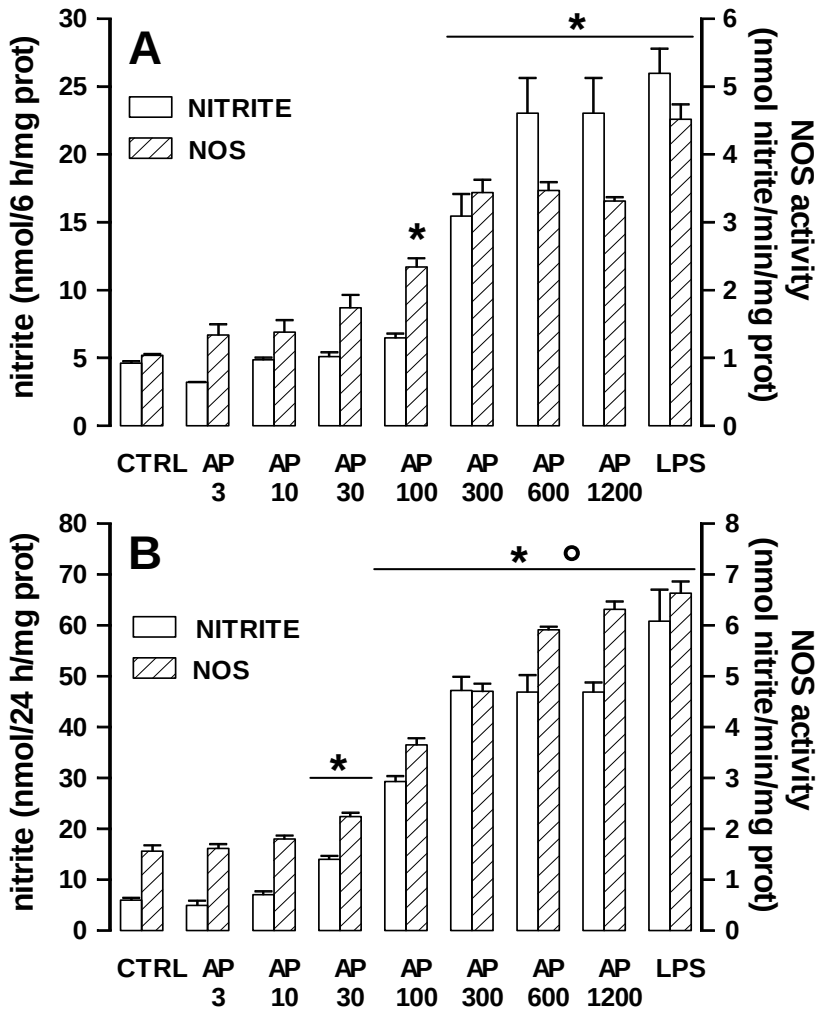

Figure 1. Effects of apocynin on nitrite levels and NOS activity in N11 cells. Cells were incubated for 6 h (**A**) and 24 h (**B**) in the absence (*CTRL*) or presence of apocynin (*AP*: 3, 10, 30, 100, 300, 600 and 1200 μM) and LPS (*LPS*: 20 μg/ml). After these incubation times, nitrite levels (*open bars*) in the extracellular medium and NOS activity (*hatched bars*) in cell lysate were measured in triplicate, as described (see Materials and Methods). Data are presented as means ± SEM (n = 3). Vs respective CTRL: * $p < 0.05$; vs. the corresponding experimental condition at 6 h: o $p < 0.05$.

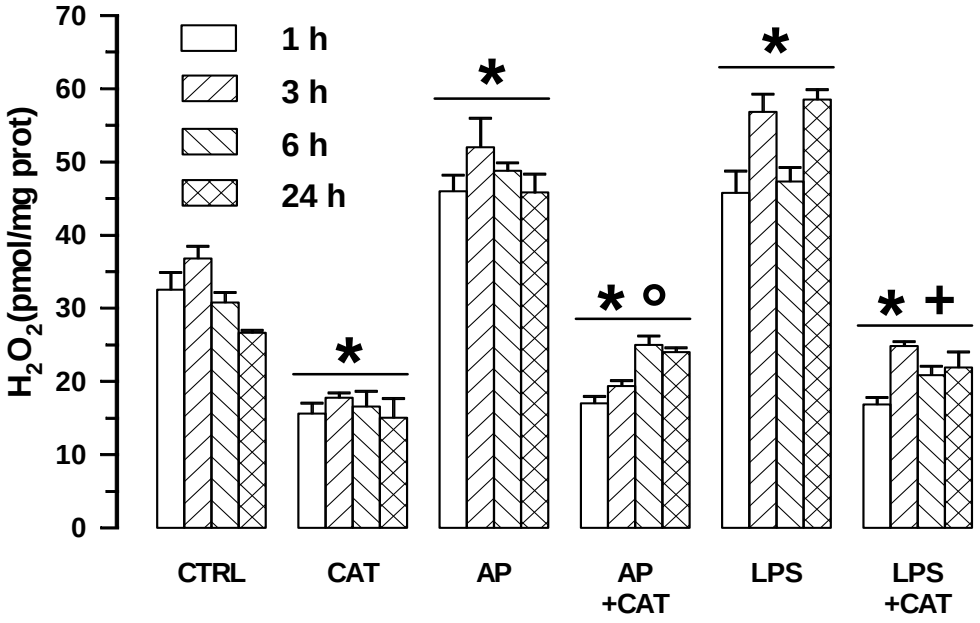

Figure 2. Effects of apocynin, LPS and catalase on the generation of H_2O_2 in N11 cells. Cells were incubated for 1, 3, 6 and 24 h in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM), catalase (*CAT*, 1000 U/ml) and/or LPS (*LPS*, 20 μg/ml) differently combined. After these incubation times, the generation of H_2O_2 was measured in triplicate, as described (see Materials and Methods). Data are presented as means ± SEM (n = 3). Vs CTRL: * $p < 0.05$; vs. AP: **o** $p < 0.05$; vs. LPS: **+** $p < 0.05$.

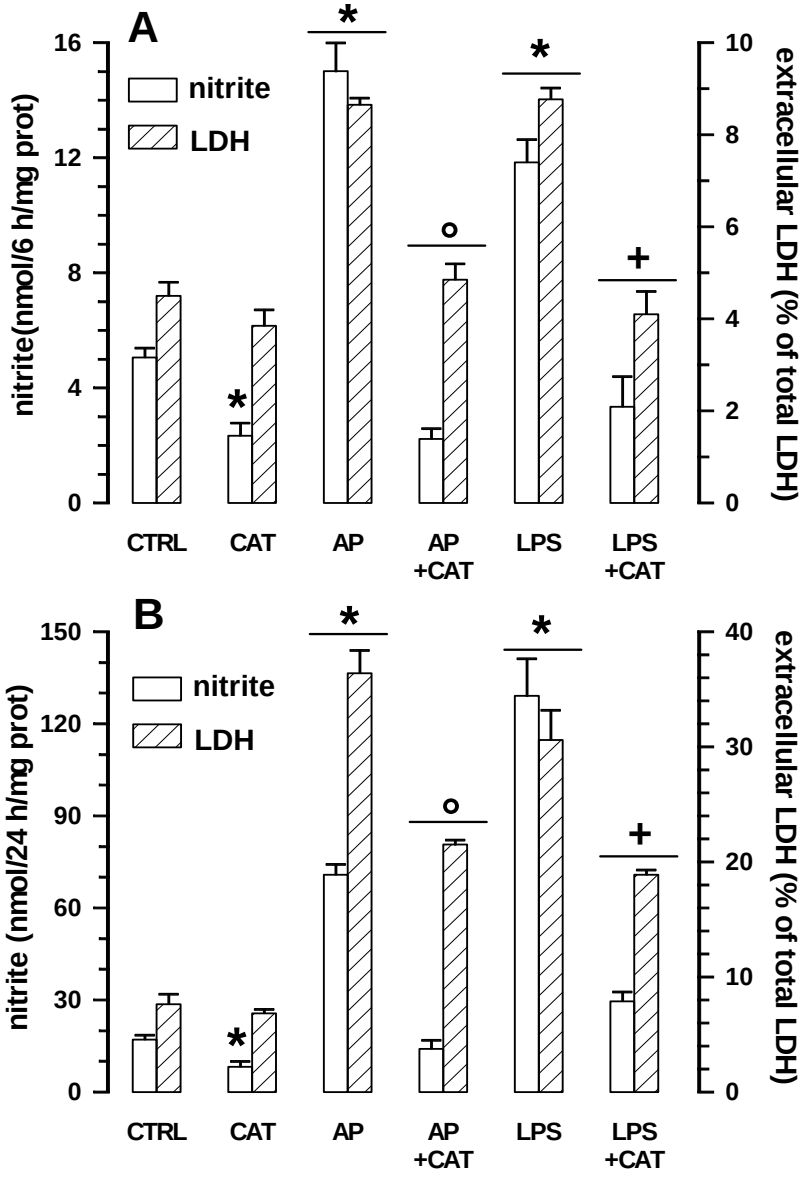

Figure 3. Effects of apocynin, LPS and catalase on the level of nitrite and the release of LDH in N11 cells. Cells were incubated for 6 h (**A**) and 24 h (**B**) in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM), catalase (*CAT*, 1000 U/ml) and/or LPS (*LPS*, 20 μg/ml) differently combined. After these incubation times, nitrite levels (*open bars*) and release of LDH (*hatched bars*) in the extracellular medium were measured in triplicate, as described (see Materials and Methods). Extracellular LDH was expressed as percentage of total (intracellular + extracellular) LDH activity per dish. Apocynin did not modify per se the activity of purified LDH (not shown). Data are presented as means ± SEM (n = 3). Vs CTRL: * $p < 0.05$; vs. AP: **o** $p < 0.05$; vs. LPS: **+** $p < 0.05$.

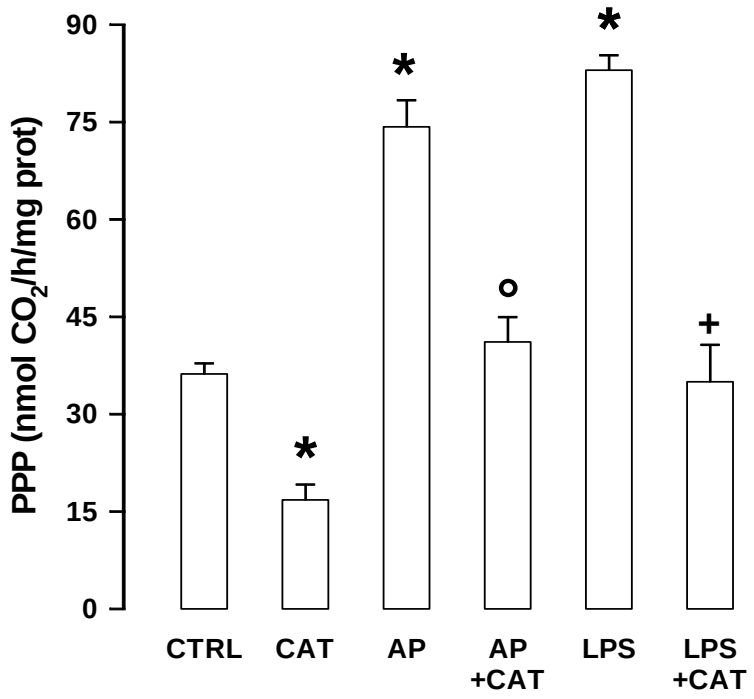

Figure 4. Effect of apocynin and LPS on pentose phosphate pathway (PPP) activity in N11 cells. Cells were detached and checked for PPP activity for 6 h (as described under Materials and Methods). During this time period they were incubated in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM) and LPS (*LPS*, 20 μg/ml), with or without catalase (*CAT*, 1000 U/ml). Measurements were performed in duplicate, and data are presented as means ± SEM ($n = 3$). Vs. CTRL: * $p < 0.05$; vs. AP: o $p < 0.05$; vs. LPS: + $p < 0.05$.

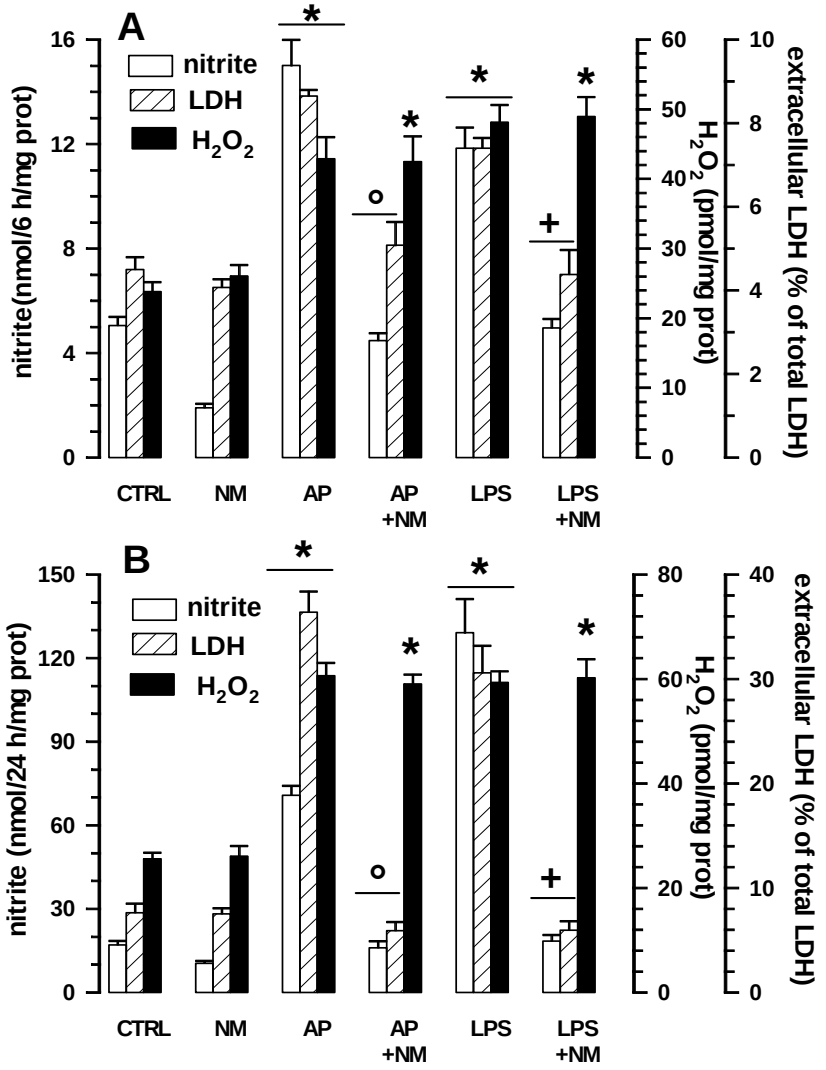

Figure 5. Effects of L-NMMA on the nitrite levels, LDH release and H_2O_2 generation elicited by apocynin and LPS in N11 cells. Cells were incubated for 6 h (**A**) and 24 h (**B**) in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM), L-NMMA (*NM*, 1 mM) and/or LPS (*LPS*, 20 μg/ml), in different combinations. At the end of the incubation times, nitrite levels (*open bars*), LDH activity in the extracellular medium (*hatched bars*) and H_2O_2 production (*solid bars*) were measured in triplicate, as reported under Materials and Methods. Extracellular LDH was expressed as percentage of total (intracellular + extracellular) LDH activity per dish. Data are presented as means ± SEM (n = 3). Vs CTRL: * $p < 0.05$; vs. AP: **o** $p < 0.05$; vs. LPS: **+** $p < 0.05$.

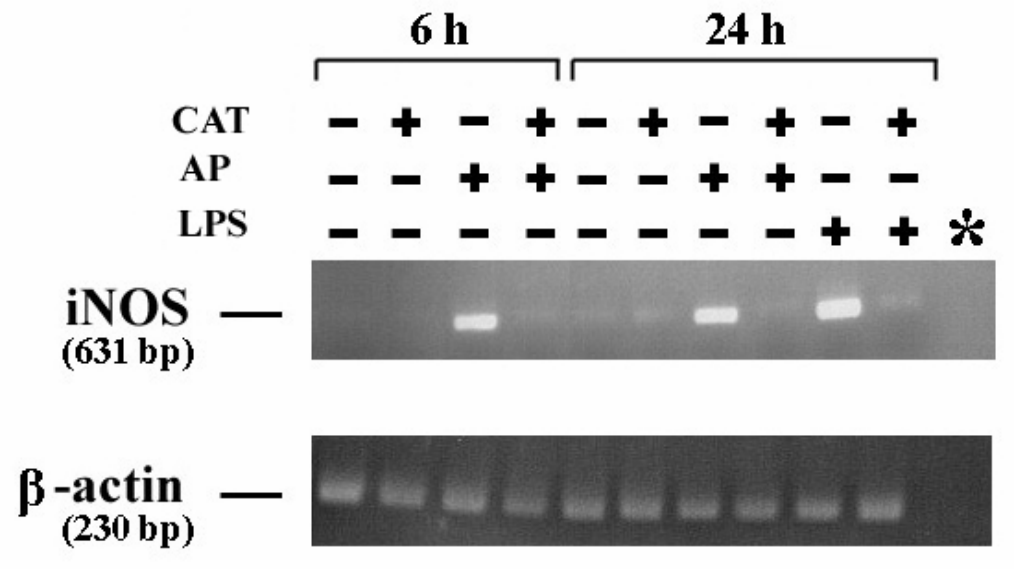

Figure 6. RT-PCR detection of iNOS and β-actin mRNA in N11 cells. Cells were incubated for 6 h and/or 24 h in the absence (control, first lane) or presence of apocynin (*AP*, 300 μM), catalase (*CAT*, 1000 U/ml) and LPS (*LPS*, 20 μg/ml), alone or in different combinations; then total RNA was extracted and RT-PCR was performed as described under Materials and Methods. Blank was performed with bisdistilled water (*) in place of cellular extracts. The level of β-actin mRNA, the product of an housekeeping gene, was measured to check the RT-PCR efficiency. The figure is representative of three experiments with similar results.

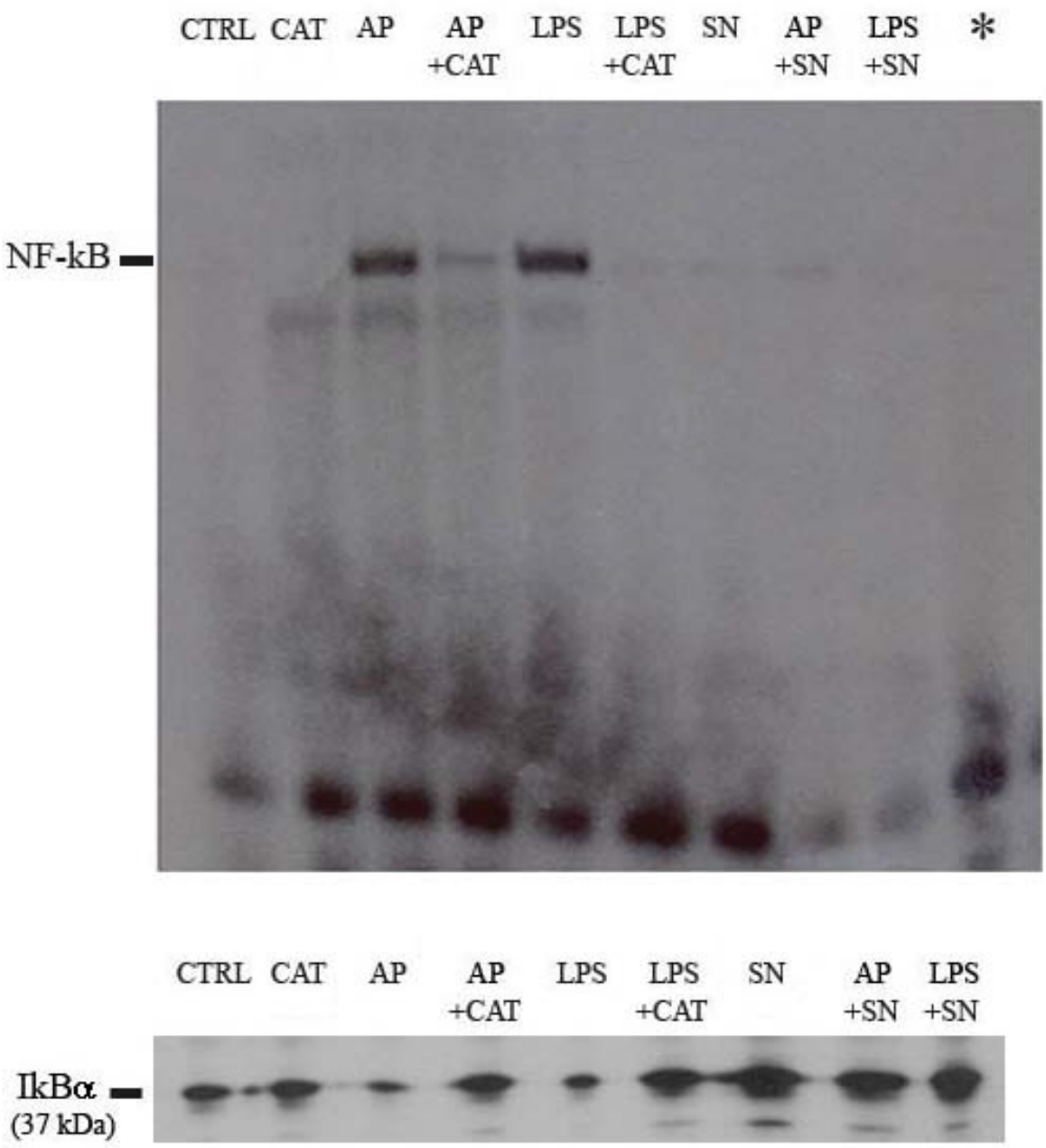

Figure 7. Effects of apocynin, LPS and catalase on the NF-kB signalling pathway in N11 cells. Cells were incubated for 6 h in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM) or LPS (*LPS*, 20 μg/ml), alone or together with catalase (*CAT*, 1000 U/ml) or the NF-kB inhibitor SN50 (*SN*, 25 μM). Each figure is representative of three experiments with similar results. **Upper panel.** EMSA detection of NF-kB was performed on nuclear extracts as described under Materials and Methods. In each experiment one lane was loaded with bisdistilled water (*) in place of cellular extracts. **Lower panel.** Western blot detection of IkBα was performed on whole cellular extracts, using an anti-IkBα antibody (see Materials and Methods for details).

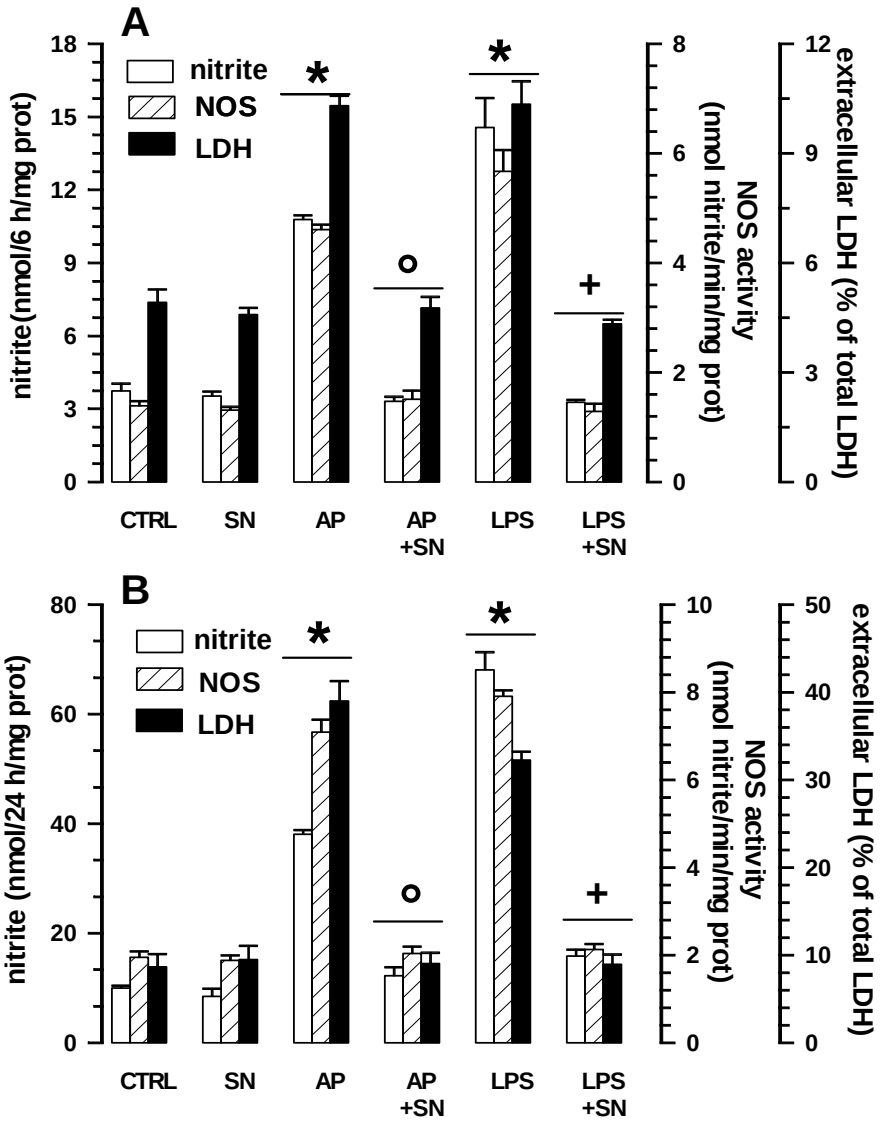

Figure 8. Effects of SN50 on the nitrite levels, NOS activity and LDH release elicited by apocynin and LPS in N11 cells. Cells were incubated for 6 h (**A**) and 24 h (**B**) in the absence (*CTRL*) or presence of apocynin (*AP*, 300 μM), SN50 (*SN*, 25 μM) and/or LPS (*LPS*, 20 μg/ml), in different combinations. At the end of the incubation times, nitrite levels (*open bars*), intracellular NOS activity (*hatched bars*) and LDH activity in the extracellular medium (*solid bars*) were measured in triplicate, as reported under Materials and Methods. Extracellular LDH was expressed as percentage of total (intracellular + extracellular) LDH activity per dish. Data are presented as means ± SEM (n = 3). Vs CTRL: * $p < 0.05$; vs. AP: **o** $p < 0.05$; vs. LPS: **+** $p < 0.05$.