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Propofol Attenuates Peroxynitrite-mediated DNA Damage and Apoptosis in Cultured Astrocytes

An Alternative Protective Mechanism

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Background: **The concentration of peroxynitrite in the brain increases after central nervous system injuries. The authors hypothesized that propofol, because of its particular chemical structure, mitigates the effects of peroxynitrite-mediated oxidative stress and apoptosis by the induction of heme oxygenase (HO)-1 in primary cultured astroglial cells.**

Methods: **Primary cultured astroglial cells were incubated for 18 h with a known peroxynitrite donor (3 mM SIN-1) in the presence or absence of propofol (40 μM, 80 μM, 160 μM, and 1 mM). The protective effects of propofol were evaluated by 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide cytotoxicity assay, lactic dehydrogenase release, DNA ladderization by Comet assay, and caspase-3 activation by Western blot analysis.**

Results: **Appropriate propofol concentrations (ranging from 40 μM to 1 mM) significantly increased HO-1 expression and attenuated SIN-1–mediated DNA ladderization and caspase-3 activation. The protective effects of propofol were mitigated by the addition of tin mesoporphyrin, a potent inhibitor of HO activity. The addition of a specific synthetic inhibitor of nuclear factor κB abolished propofol-mediated HO-1 induction, suggesting a possible role of this nuclear transcriptional factor in our experimental conditions.**

Conclusions: **The antioxidant properties of propofol can be partially attributed to its scavenging effect on peroxynitrite as well as to its ability to increase HO-1 expression at higher concentrations, a property that might be relevant to neuroprotection during anesthesia.**

PEROXINITRITE, which results from the reaction between nitric oxide and superoxide anion, is an oxidant that can react with proteins, lipids, carbohydrates, and nucleic acids. Peroxynitrite has been reported to play a role in cell or tissue damage associated with circulatory shock,[1] inflammation,[2] ischemia–reperfusion injury,[3] and respiratory[4] diseases as well as in neurodegenerative disorders such as Alzheimer disease[5] and Parkinson disease.[6] Peroxynitrite is involved in the nitration of tyrosyl residues in proteins; this reaction can alter cell signaling processes and plays a pivotal role in the mechanism of apoptosis.[7]

Propofol is an intravenous sedative–hypnotic agent commercially introduced in the United States in 1989 by Zeneca Pharmaceuticals. It was the first of a new class of intravenous anesthetic agents: the alkylphenols. It is indicated for induction and maintenance of general anesthesia as well as for sedation of intubated, mechanically ventilated adults in the intensive care unit. Propofol is characterized by a phenolic structure similar to that of α-tocopherol and presents antioxidant properties that have been demonstrated *in vitro* and *in vivo*. Propofol has been reported to inhibit lipid peroxidation in various experimental models,[8,9] to protect cells against oxidative stress, and to increase the antioxidant capacity of plasma in humans.[10,11] Recently, Mathy-Hartert *et al.*[12] demonstrated that propofol reacts with peroxynitrite, leading to the formation of a propofol-derived phenoxyl radical, and has therefore been hypothesized to be a peroxynitrite scavenger. The majority of *in vitro* and *in vivo* studies conducted so far have attributed the protective effect of bioactive polyphenols to their chemical reactivity toward free radicals and their capacity to prevent the oxidation of important intracellular components. However, Scapagnini *et al.*[13] suggested a possible novel aspect in the mode of action of these compounds, *i.e.*, the ultimate stimulation of the heme oxygenase (HO)-1 pathway is likely to account for the established and powerful antioxidant/antiinflammatory properties of these polyphenols.

Heme oxygenase isoforms catalyze the conversion of heme to carbon monoxide and bilirubin with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration.[14,15] HO is the physiologic pathway of heme degradation and, consequently, plays a critical role in the regulation of cellular heme-dependent enzyme concentrations.[16] To date, two HO isoforms have been shown to be catalytically active in heme degradation, and each is encoded by a different gene.[15,17] HO-1, the inducible isoform, is found ubiquitously in all organs with the exception of the adult brain and is rapidly and transiently expressed after various stimuli including peroxynitrite. Our recent study demonstrates that HO-1 expression undergoes modification during astroglial cell differentiation, reaching maximal values at 14 days *in vitro*[18] and decreasing progressively

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after 21 days *in vitro*. In contrast, HO-2 is responsible for most HO constitutive activity and does not undergo modifications during astroglial differentiation.

Because of propofol phenolic chemical structure, the current study was undertaken to evaluate its scavenger activity and effects on the HO system and how this may impact peroxynitrite-mediated oxidative stress and apoptosis in primary astroglial cell cultures.

Materials and Methods

Cell Cultures and Animals

All efforts were made to minimize the animals' suffering and the number of animals used. All experiments were approved and conformed to the guidelines of the Ethical Committee of the University of Catania, Italy. Primary cultures of astrocytes were prepared from cerebral cortex of newborn albino rats (1- to 2-day-old Wistar strain) as previously reported.[19] Briefly, after dissection and mechanical dissociation of cerebral tissues, isolated cells were suspended in Dulbecco's Modified Eagle's Medium supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (50 μg/ml), and penicillin (50 U/ml). Cells were plated in Falcon Petri dishes at a density of 6×10^5 cells in 35 mm of diameter and 3×10^6 cells in 100 mm of diameter, maintained at 37°C in a 5% CO_2–95% air humidified atmosphere for 2 weeks. Medium of cell cultures was replaced twice a week. Astroglial cell purity was performed by immunofluorescence staining for glial fibrillary acidic protein as previously reported.[20]

Materials

Cell culture medium and sera were obtained from Life Technologies Ltd. (Milano, Italy). Monoclonal HO-1 antibody was from Stressgen Biotechnologies (Victoria, BC, Canada). Secondary horseradish peroxidase–conjugated anti-mouse antibody and synthetic nuclear factor (NF) κB inhibitor (SC-3060) was from Santa Cruz Biotechnology (Santa Cruz, CA). The Enhanced Chemiluminescence System for developing immunoblots and nitrocellulose membranes was purchased from Amersham (Milano, Italy). Intralipid (20% emulsion, phospholipid stabilized soybean oil) and 3-morpholino sydnonimine-hydrochloride (SIN-1) were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were purchased from Merck (Frankfurt, Germany).

Propofol and SIN-1 Treatment

Astrocytes at 13 days *in vitro* were treated with 1, 2, or 3 mM SIN-1 for 18 h, in the presence or absence of 40 μM, 80 μM, 160 μM, or 1 mM propofol or a corresponding amount of Intralipid (the vehicle solution of propofol in Diprivan® [Astrazeneca, Milan, Italy]). To evaluate the role of HO, tin mesoporphyrin (SnMP; 10 μM), a potent inhibitor of HO activity, was added to the culture medium 30 min before SIN-1 exposure. To verify the involvement of NF-κB, astroglial cell cultures were also pretreated for 30 min with 0.2 μl/ml of a synthetic peptide, specific inhibitor of NF-κB. Our preliminary results by using electrophoretic mobility shift assay showed that maximal NF-κB inhibition was obtained at the tested concentration. Four replicates were performed for each sample.

MTT Bioassay

To monitor cell viability, astrocytes were set up 60 × 104 cells/well of a 96-multiwell flat-bottomed 200-μl microplate.[21] Cells were incubated at 37°C in a humidified 5% CO_2–95% air mixture. At the end of treatment time, 20 μl 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), 0.5%, in phosphate buffer saline were added to each microwell. After 1 h of incubation with the reagent, the supernatant was removed and replaced with 200 μl dimethyl sulfoxide. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at 570 nm.

Lactic Dehydrogenase Release

Lactic dehydrogenase (LDH) release was measured to evaluate the presence of cell necrosis as a result of cell disruption subsequent to membrane rupture. LDH activity was measured spectrophotometrically in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during pyruvate-lactate transformation.[22] The percentage of LDH release was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

DNA Analysis by Comet Assay

The presence of DNA fragmentation was examined by single-cell gel electrophoresis (Comet assay), according to Singh *et al.*[23] Briefly, $0.8–1 \times 10^5$ cells were mixed with 75 μl low melting agarose, 0.5%, and spotted on slides. The "minigels" were maintained in lysis solution (1% N-laurosil-sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10) for 1 h at 4°C and denatured in a buffer (300 mM NaOH, 1 mM Na_2EDTA) for 20 min and finally electrophoresed in the same buffer at 13 V for 60 min. At the end of the run, the minigels were neutralized in 0.4 M Tris-HCl, pH 7.5, stained with 100 μl ethidium bromide (2 μg/ml) for 3 min and scored using a Leika fluorescence microscope (Leika, Wetzlar, Germany) interfaced with computer software (Cayman Sarin, Florence, Italy). In this way, DNA damage can be analyzed and quantified by measuring the tail moment

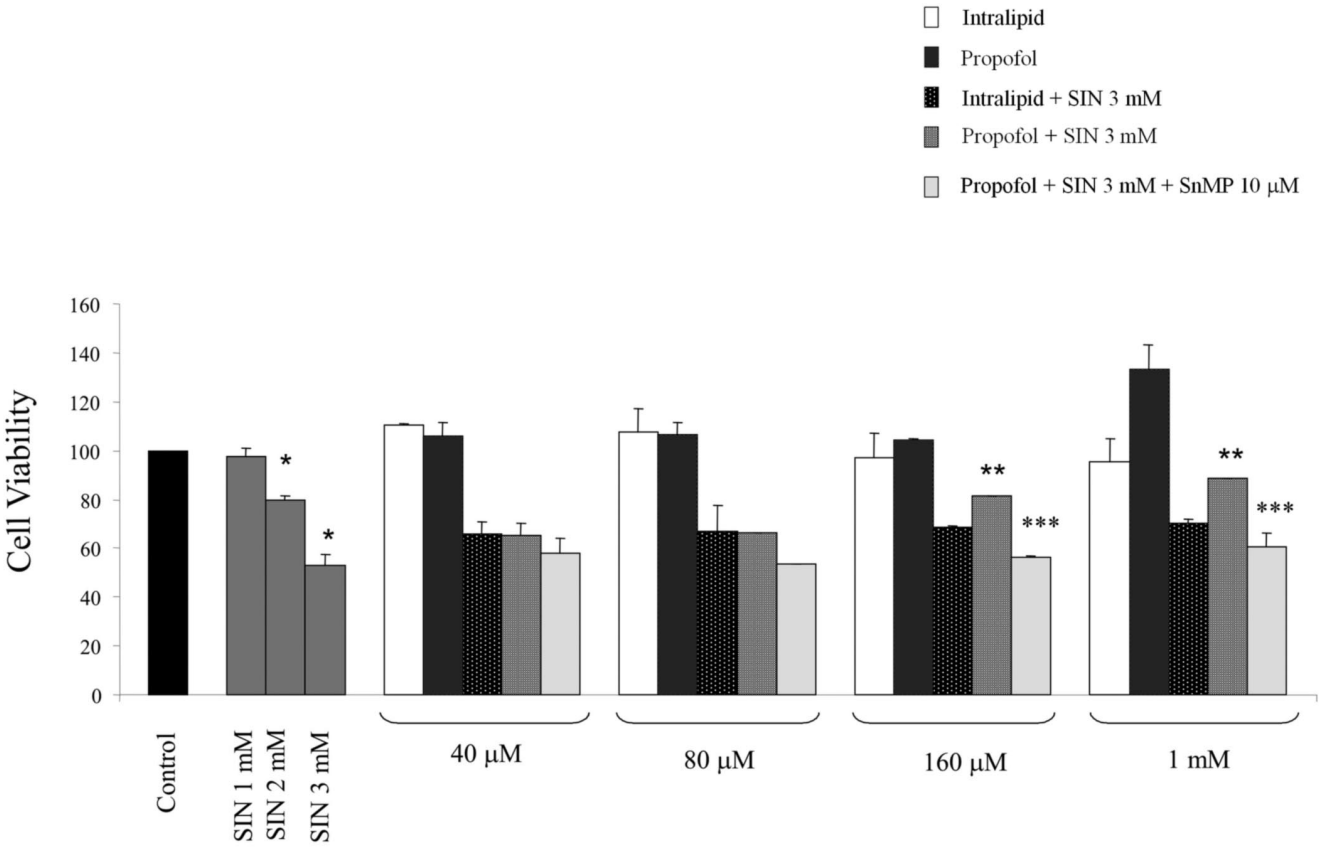

Fig. 1. Cell viability in rat astrocytes after treatment with SIN-1, propofol, and Intralipid at different concentrations. Values are mean ± SD of four experiments in duplicate. * Statistically significant *versus* control, $P < 0.001$; ** statistically significant *versus* 3 mM SIN-1, $P < 0.001$; * statistically significant *versus* propofol + 3 mM SIN-1, $P < 0.001$. SnMP = tin mesoporphyrin.**

(TMOM) expressed as the product of tail head distance and percentage of the fragmented DNA.

Western Blotting

Astrocytes were harvested using cell lysis buffer as previously described.[18] The lysate was collected for Western blot analysis, and protein expression was visualized by immunoblotting with antibodies against HO-1, HO-2, or caspase-3. Briefly, 30 μg lysate supernatant was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated overnight with 5% milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBST) buffer at 4°C. After washing with TBST, the membranes were incubated with a 1:1,000 dilution of anti–HO-1, anti–HO-2 or anti–caspase-3 antibody for 1 h at room temperature with constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase–conjugated antimouse immunoglobulin G (Amersham) for HO-1 and caspase-3 at a dilution of 1:2,000 or horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Amersham) for HO-2 at a dilution of 1:5,000. Chemiluminescence detection was performed using an Enhanced Chemiluminescence Detection kit according to the manufacturer's instructions.

Statistical Analysis

One-way analysis of variance followed by Bonferroni *t* test was performed to estimate significant differences among groups. Data were reported as mean ± SD, and differences between groups were considered to be significant at $P < 0.005$.

Results

Effects of Propofol on Primary Cultured Astrocytes Subjected to SIN-1–induced Cytotoxicity

The effects of propofol on astrocytes cytotoxicity after treatment with different SIN-1 concentrations are shown in figure 1. The effects of equivalent amounts of Intralipid were tested under the same conditions. Treatment of primary cultured astroglial cells with 1, 2, or 3 mM SIN-1 for 18 h resulted in a dose-dependent increase in cytotoxicity. The addition of propofol significantly reduced SIN-1–induced cytotoxicity. Intralipid alone did not prevent SIN-1–mediated cytotoxicity. Pretreatment with SnMP, a potent inhibitor of HO activity, resulted in

Table 1. Lactic Dehydrogenase Release in Untreated and Treated Cultured Astrocytes for 18 h with SIN-1, Propofol, or Intralipid

Treatment	% Lactic Dehydrogenase Released
Control	12.10 ± 0.004
1 mM SIN-1	10.60 ± 0.001
2 mM SIN-1	9.90 ± 0.023
3 mM SIN-1	13.03 ± 0.016
40 μM Propofol	10.73 ± 0.001
Intralipid	9.79 ± 0.003
80 μM Propofol	11.55 ± 0.012
Intralipid	10.85 ± 0.008
160 μM Propofol	11.79 ± 0.010
Intralipid	13.15 ± 0.011
1 mM Propofol	13.3 ± 0.038
Intralipid	12.5 ± 0.0057

Values are presented as mean ± SD of three different experiments.

a significant increase in SIN-1–mediated cytotoxicity and mitigated the protective effect of propofol at 160 μM and 1 mM where the expression of HO-1 was maximal, thus suggesting a possible role of HO in the propofol antioxidant pathway. Furthermore, pretreatment with SnMP did not show increased cytotoxicity when compared with 3 mM SIN-1–treated cells. The addition of SnMP alone did not result in a significant increase in cytotoxicity (data not shown). LDH release in treated cells did not differ from control after exposure to SIN-1, propofol, or Intralipid (table 1), thus suggesting that SIN-1 induced apoptotic cell death.

Effect of Propofol on DNA Damage by Peroxynitrite

DNA ladderization was evaluated in astroglial cells treated with SIN-1 to assess the effect of propofol on peroxynitrite DNA damage, quantified by image analysis to obtain a TMOM. No significant changes were observed after treatment with propofol or Intralipid when compared with untreated cell cultures (figs. 2A and B). By contrast, treatment with SIN-1 resulted in a dose-dependent increase in the TMOM when compared to control ($P < 0.001$; figs. 2A and C). As shown in figures 2A, D, E, F, and G, the addition of propofol to SIN-1 (3 mM)–treated astrocytes resulted in a decrease in the TMOM ($P < 0.001$), and this effect was mitigated by the addition of SnMP, thus suggesting a possible role of HO in the protective effect of propofol. Interestingly, the addition of Intralipid to SIN-1 (3 mM)–treated astroglial cells was also able to reduce the TMOM when compared with control, thus suggesting that the protective effect of propofol on DNA may be related also to Intralipid itself.

Western Blot Analysis

HO-1 and HO-2. Astrocytes were examined for expression of HO-1 and HO-2 proteins by Western blot analysis. The results of three representative experiments are reported in figures 3A and B. Astroglial cells showed basal expression of HO-1 protein and a dose-dependent increase after treatment with SIN-1 as compared with control. Interestingly, propofol increased HO-1 levels at concentrations of 160 μM and 1 mM. By contrast, use of Intralipid did not show a significant increase in HO-1 protein expression. Treatment with SIN-1, propofol, or Intralipid did not significantly modify HO-2 protein, the constitutive isoform of the enzyme. The addition of SnMP, a known inhibitor of HO activity and a transcriptional activator of the HO-1 gene,[24] resulted in up-regulation of HO-1 protein but had no effect on HO-2 protein (fig. 3A). To further elucidate the molecular mechanism by which propofol (160 μM and 1 mM) induces HO-1 expression, we used a synthetic inhibitor of NF-κB, a known nuclear transcription factor of HO-1. The addition of NF-κB inhibitor to propofol treated astroglial cells completely reversed HO-1 induction, thus suggesting that propofol induces HO-1 expression by activation of the NF-κB pathway (fig. 3C).

Caspase-3 Activation. To investigate the possibility that cytotoxic injury produced by SIN-1 may be associated with apoptotic cell death, we used the Western blot method to detect activation of caspase-3 in response to peroxynitrite, as previously described.[25] SIN-1 induced dose-dependent increases in the expression of activated caspase-3, which was exacerbated by the addition of SnMP (fig. 4A), thus further confirming that SIN-1 cell death is apoptotic.

Treatment of cultures with 40, 80, and 160 μM propofol or Intralipid alone did not increase caspase-3 activation (fig. 4B). Surprisingly, treatment with 1 mM propofol resulted in a significant increase in caspase-3 activation despite the strong induction of HO-1 protein. To evaluate the possible antiapoptotic role of propofol, we added this compound to SIN-1–treated astroglial cells. Figure 4C shows a dose-dependent decrease in SIN-1–mediated caspase-3 activation, peaking at 160 μM and 1 mM where the expression of HO-1 was maximal. The addition of SnMP completely reversed the antiapoptotic effects of propofol at 160 μM and 1 mM.

Discussion

This study shows that propofol protects astroglial cells in a dose-dependent manner against peroxynitrite-mediated cytotoxicity, DNA ladderization, and apoptosis, and these effects are in part mediated by induction of HO-1. To investigate the protective effect of propofol against peroxynitrite-induced cytotoxicity, we used SIN-1, a peroxynitrite donor, because it produces both nitric oxide and superoxide anion on decomposition in aqueous solution.

We demonstrated that SIN-1 cytotoxicity in astrocytes is significantly attenuated by propofol in a dose-dependent manner. This protective effect should be attributed

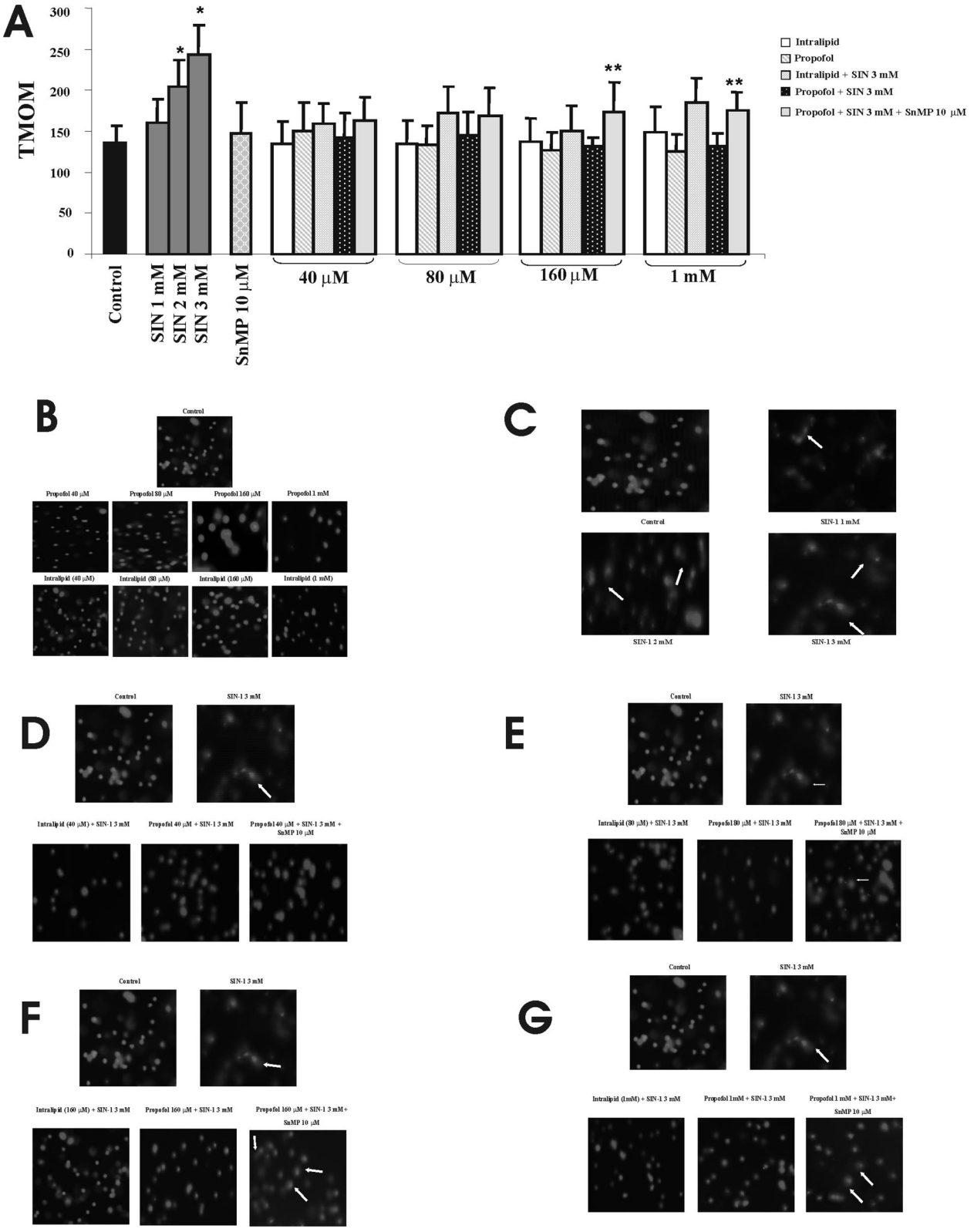

Fig. 2. Comet assay of genomic DNA of rat astrocytes. Tail moments were quantified using Scion Image software. Values are mean ± SD of four experiments performed in duplicate (*A*). * Statistically significant *versus* control, $P < 0.001$; ** statistically significant *versus* propofol + 3 mM SIN-1, $P < 0.001$). Astrocytes were treated for 24 h with propofol or a corresponding amount of Intralipid (40 μM, 80 μM, 160 μM, and 1 mM) (*B*) and SIN-1 at different concentrations (1, 2, and 3 mM) (*C*). *D*, *E*, *F*, and *G* show the protective effects of propofol and Intralipid in SIN-1 (3 mM)–treated astroglial cells. *Arrows* indicate comet tails. SnMP = tin mesoporphyrin.

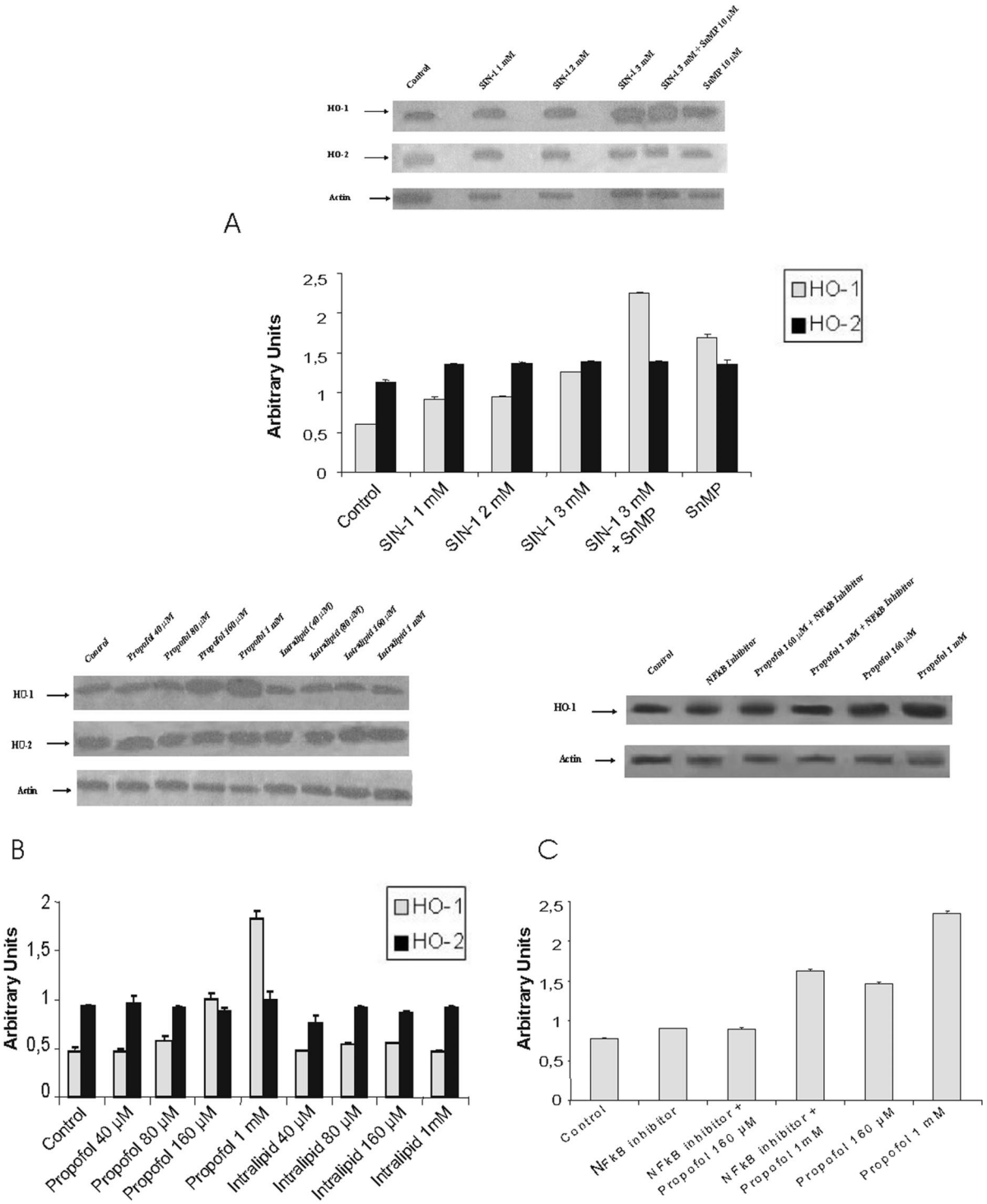

Fig. 3. Heme oxygenase (HO)-1 and HO-2 protein expression in astroglial cell cultures treated with SIN-1 (1, 2, and 3 mM) (*A*) and propofol (40 μM, 80 μM, 160 μM, and 1 mM) or Intralipid (*B*) was visualized by immunoblotting with antibodies against HO-1 or HO-2. The cells were also pretreated for 30 min with a synthetic inhibitor of nuclear factor (NF) κB (*C*). Densitometric analysis was performed after normalization with actin. Blots shown are representative of Western blot analysis from four separate experiments. SnMP = tin mesoporphyrin.

to propofol itself and not to the vehicle solution of propofol, because Intralipid alone did not prevent SIN-1–mediated cytotoxicity. This result is consistent with recent studies showing that Intralipid interacts with reactive oxygen species to a lesser degree than propofol[26] and shows only a weak protective effect after oxidative stress induced by either hydrogen peroxide or myeloperoxidase.[12] Our data also showed that the cytotoxic effect induced by the treatment with SIN-1 was not due to cell disruption subsequent to membrane breakdown;

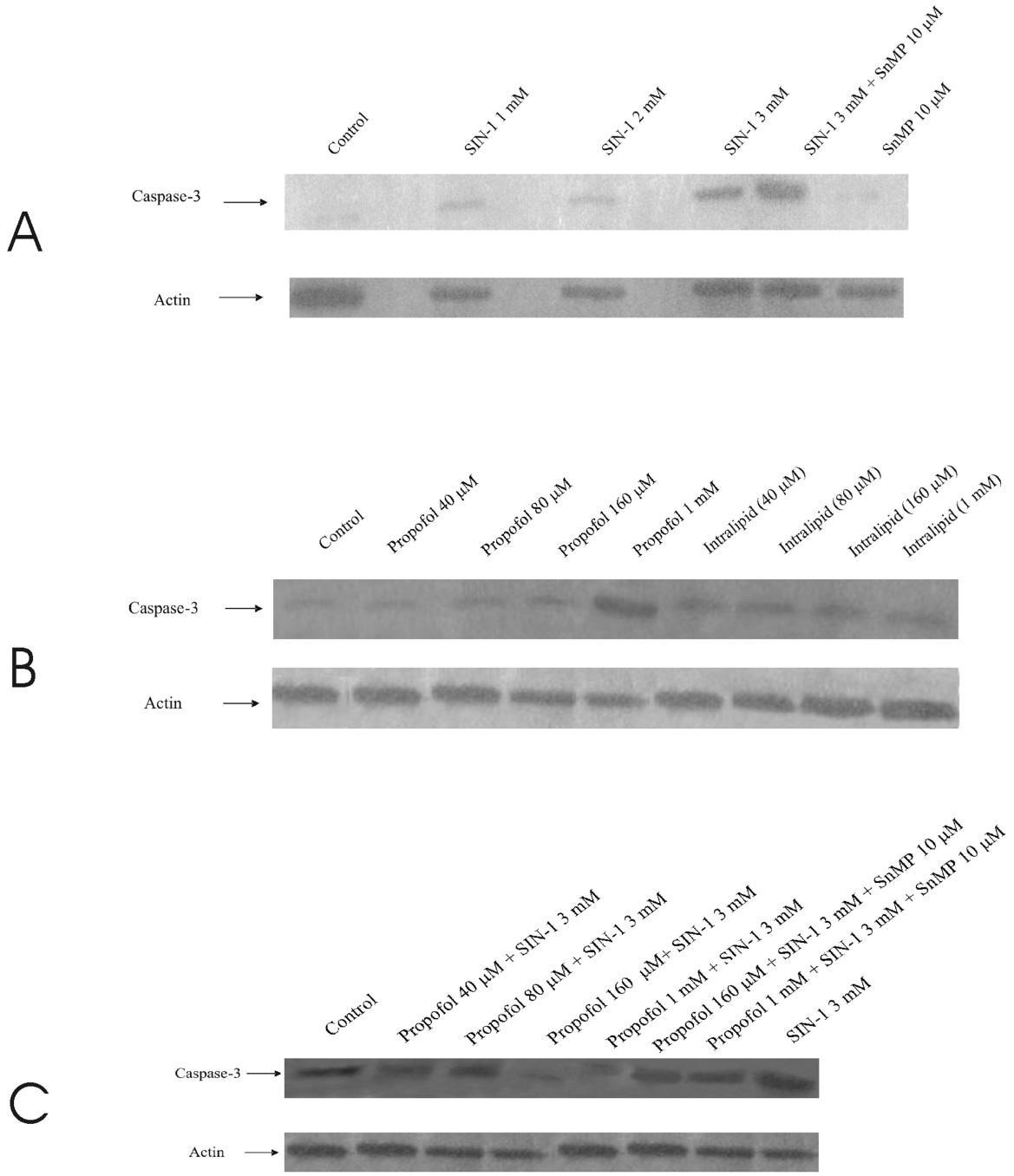

Fig. 4. Western blot analysis demonstrating caspase-3 activation in astroglial cells induced by SIN-1 (1, 2, and 3 mM) (*A*) and propofol (40 μM, 80 μM, 160 μM, and 1 mM) or Intralipid exposure (*B*). Astrocytes were exposed to SIN-1 (3 mM) for 18 h in the presence or absence of propofol (40 μM, 80 μM, 160 μM, and 1 mM) or Intralipid (*C*). The activation of caspase-3 was assessed by the presence of a 17-kd fragment derived from the cleavage of proenzyme caspase-3. Immunoblots are representative of four independent experiments. SnMP = tin mesoporphyrin.

in fact, no significant LDH release was found in SIN-1–treated astrocytes when compared with control cells, whereas we observed strong caspase-3 activation.

We also evaluated DNA damage by Comet assay, widely considered a versatile and highly effective tool in biomonitoring DNA integrity.[27] Our results indicate that peroxynitrite induced DNA damage in a dose-dependent manner without an increase in cellular membrane breakage. These results suggest an apoptotic cell death characterized by formation of apoptotic bodies without membrane disruption, chromatin condensation, or oligonucleosomal fragmentation of chromosomal DNA.[28] Recent data[29,30] indicate that only cells with high TMOM values can be considered apoptotic. In fact, SIN-1 induces high values of TMOM, which were attenuated by the addition of propofol in a dose-dependent manner.

The protective effects of propofol on DNA damage were reversed by the addition of SnMP, thus suggesting HO as a protective pathway. Interestingly, the addition of Intralipid to SIN-1 (3 mM)–treated astroglial cells also reduced the TMOM when compared with control.

We further evaluated the protective effect of propofol on peroxynitrite-mediated apoptosis as it occurs in photoreceptors cells[31] and in cerebral vascular muscle cells.[32] Here, we observed activation of caspase-3 in astrocytes after exposure to SIN-1, in accord with previous findings on rat spinal cord.[33] In the current study, we demonstrate that SIN-1 induced caspase-3 activation in a dose-dependent manner, and propofol treatment (160 μM and 1 mM) was able to attenuate SIN-1–mediated apoptosis. In addition, we showed that SnMP, a potent inhibitor of HO activity, can partially reverse the protective effects of propofol, thus providing further evidence for HO-1 as a possible antiapoptotic pathway of propofol. Surprisingly, treatment with 1 mM propofol resulted in a significant increase of caspase-3 activation despite the strong induction of HO-1. It is possible to hypothesize that endogenous carbon monoxide is toxic at high concentrations. Consistent with this hypothesis is our preliminary result showing that treatment with high concentrations of carbon monoxide–releasing molecules produces increased cytotoxicity and apoptosis in both astroglial and endothelial cells. Furthermore, propofol-mediated HO-1 induction may explain in part some effects of propofol on important heme proteins, such as cytochrome P450 (CYP450) and cyclooxygenase. In this regard, Yang *et al.*[34] showed that propofol may be a potential CYP450 inhibitor because it can significantly inhibit 3A4 isoenzyme activity without significantly affecting the protein expression. In addition, Ogawa *et al.*[35] showed that propofol reduces cyclooxygenase activity, as measured by prostaglandin production. Recently, Li Volti *et al.*[36] showed that pharmacologic- and retroviral-mediated up-regulation of HO-1 reduces intracellular heme content, and this may account for reduced cyclooxygenase activity. Because heme functions as the prosthetic group in both CYP3A4 and cyclooxygenase, propofol-mediated HO-1 induction may be responsible for their reduced activity by decreasing intracellular heme content. In addition, it is important to note that the gene encoding for HO-1 protein contains an NF-κB binding site, which can be selectively recognized by the ubiquitous transcriptional factor NF-κB.[37] Addition of a synthetic NF-κB inhibitor completely reversed propofol-mediated HO-1 expression. However, experiments designed to verify whether NF-κB activation is required for propofol-mediated HO-1 gene expression are now warranted.

Whether the cytoprotective effect of propofol against peroxynitrite-mediated injury demonstrated here *in vitro* accurately translates to a therapeutic effect in patients remains uncertain. Nevertheless, this study is the first to postulate HO-1 as a possible new cytoprotective pathway of propofol against peroxynitrite-mediated injury.

In conclusion, our studies demonstrated that propofol not only has an antiapoptotic effect, reduces cytotoxicity, and prevents DNA cleavage in peroxynitrite injury, but also utilizes HO-1 in exerting its antiapoptotic effect in primary astroglial cell cultures, thus providing a new and powerful strategy for neuroprotection during anesthesia.

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