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Synergistic antiglioma action of hyperthermia and nitric oxide

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Abstract

To explore combined antiglioma effect of nitric oxide (NO) and hyperthermia, the rat C6 and human U251 glioma cells were exposed to NO-releasing agents sodium nitroprusside(SNP), S-nitrosoglutathione or PAPA-NONOate, followed by hyperthermia (1 h, 43 °C). While each treatment alone showed only moderate efficiency, a synergistic cytotoxicity of NO donors and hyperthermia was clearly demonstrated by crystal violet and MTT cytotoxicity assays. The flow cytometric analysis with the appropriate reporter fluorochromes confirmed that hyperthermia and SNP cooperated in inducing oxidative stress, mitochondrial depolarization, caspase activation and DNA fragmentation, leading to both necrotic and caspase-dependent apoptotic cell death. The acridine orange staining of intracellular acidic compartments revealed that SNP completely blocked hyperthermia-induced autophagy, while the inhibition of autophagy by 3-methyl adenine mimicked SNP-triggered oxidative stress, caspase activation and cell death in hyperthermia-exposed cells. Therefore, the synergistic cytotoxicity of SNP and hyperthermia could result from NO-mediated suppression of protective autophagic response in glioma cells.

Keywords: Hyperthermia; Nitric oxide; Glioma; Oxidative stress; Mitochondrial depolarization apoptosis; Necrosis; Autophagy

1. Introduction

High temperatures can damage and kill cancer cells both in vitro and in vivo, usually with minimal injury to normal tissues. While the exact mechanisms of heat-induced cell death are still not entirely clear, it appears that the most important effects occur at the plasma membranes (alteration of membrane lipids, membrane transport and receptors), proteins (protein denaturation, unfolding, aggregation), nucleic acids (chromosomal aberrations, mitotic dysfunctions, inhibition of repair of DNA damage) and cytoskeleton (cell rounding and blebbing) (Lepock, 2003). Heat-stress potentiates the effects of chemotherapy and ionizing radiation through increased intracellular drug uptake and intratumor drug concentrations, enhanced DNA damage, and improved tissue oxygenation resulting from an increase in blood flow (van der Zee, 2002). For these reasons, local (e.g. microwave-or magnetic nanoparticle-induced) or systemic (whole-body) hyperthermia has been regarded as potentially useful approach against cancer, particularly in conjunction with radiotherapy or chemotherapy. Among the promising targets for hyperthermic treatment are gliomas, extremely aggressive neuroectodermal tumors that represent the most common primary malignancy in human central nervous system (Giese et al., 2003). Gliomas are incurable in most of the cases, primarily due to ability to resist chemotherapy- or radiation-induced apoptosis (Giese et al., 2003). Many in vitro and in vivo studies have confirmed the potent antiglioma activity of hyperthermia, exerted either directly or in combination with various chemotherapy, radiotherapy or gene therapy regimens (Bouhon et al., 1999; Fukami et al., 2004; Hermisson et al., 2000; Jordan et al., 2006; Magin and Johnson, 1979; Naruse et al., 1986; Okamoto et al., 2001; Uesugi et al., 1998).

Nitric oxide (NO) is a small, hydrophobic and highly diffusible molecule implicated in numerous physiological and pathological

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conditions such as smooth muscle relaxation, neurotransmision, immune regulation and host defense against pathogens and tumors (Thippeswamy et al., 2006). NO is very reactive and generates a variety of secondary products such as the nitrosonium ion (NO^+), peroxynitrite ($ONOO^-$), nitrosothiols and nitroxyl anion (NO^-). These reactive nitrogen species, including NO, interact with biomolecules to cause DNA damage, lipid oxidation, protein modification and alterations in enzyme activity (Kim et al., 2002). Since NO can kill tumor cells through induction of mitochondrial damage and activation of caspase signaling pathway (Chung et al., 2001), the NO-based strategies are currently being considered for anticancer treatment. In addition to being intracelullarly produced by NO synthase enzyme family (Thippeswamy et al., 2006), NO can be generated by various NO-releasing chemicals. Sodium nitroprusside (SNP) is a clinically approved NO donor primarily used for the treatment of acute hypertension (Friederich and Butterworth, 1995), which also exerts an in vitro antiproliferative and/or cytotoxic activity toward various glioma cell lines (Bernabe et al., 2001; Blackburn et al., 1998). Moreover, it has been shown that administration of SNP to rats during local hyperthermia significantly retards the growth of subcutaneously implanted BT4An glioma (Krossnes et al., 1996; 1998). While the mechanisms of this beneficial action of SNP were not explored in detail, it was proposed that SNP-mediated vasodilatation additionally increased tumor temperature and therefore potentiated the antitumor effect of hyperthermia. However, the possibility that SNP might potentiate the effect of hyperthermia by directly acting on tumor cells has not been investigated thus far.

The aim of the present study was to examine the effect of NO donor SNP and hyperthermia on survival of glioma cells in vitro, as well as to determine the mechanisms of their possible interaction. Our data for the first time demonstrate the ability of hyperthermia and SNP-released NO to kill glioma cells in a synergistic manner, through mechanisms involving induction of mitochondrial depolarization and oxidative stress.

2. Materials and methods

2.1. Cells and cell culture

The rat glioma cell line C6 and the human glioma cell line U251 were kindly donated by Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in a HEPES (20 mM)-buffered RPMI 1640 cell culture medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 10 mM sodium pyruvate, 50 μM 2-mercaptoethanol and penicillin/streptomycin (all from Sigma, St. Louis, MO). The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA and incubated in cell culture medium in flat-bottom 96-well (1.5×10^4 cells/well) or 24-well (1×10^5 cells/well) cell culture plates (Sarstedt, Newton, NC) for the cell viability assessment or flow cytometry, respectively. Cells were rested for 24 h and then treated with NO donors SNP, S-nitroso glutathione (GSNO) or (*Z*)-1-(*N*-(3-aminopropyl)-*N*-(*n*-propyl)amino)diazen-1-ium 1,2-diolate (PAPA-NONOate) (all from Sigma, St. Louis, MO), as described in figure legends. In some experiments, the autophagosome formation inhibitor 3-methyladenine (Blommaart et al., 1997) was used instead of NO donors to block autophagy. After 30 min, the cells were exposed to hyperthermia (1 h at 43 °C in an incubator with a humidified atmosphere and 5% CO_2), while control cells were maintained at 37 °C. Following hyperthermia, cells were incubated for additional 6 h (oxidative stress, autophagy), 18 h (mitochondrial depolarization, caspase activation) or 24 h (cell viability, cell cycle, apoptosis/necrosis).

2.2. Cell viability

The number of adherent, viable cells was assessed using a crystal violet assay, while mitochondrial dehydrogenase activity, as another indicator of cell viability, was determined by mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan. Both tests were performed exactly as previously described (Harhaji et al., 2007a,b; Miljkovic et al., 2004) and the results were presented as % of the control value (untreated cells). The degree of interaction between the two treatments was evaluated using the following equation: $\alpha = SF^{SNP} \times SF^{HT}/SF^{SNP+HT}$, where SF^{SNP} and SF^{HT} represent surviving fractions after SNP and hyperthermia treatments, respectively, and SF^{SNP+HT} is the surviving fraction following combined treatment. In this analysis, $\alpha=1$ indicates an additive effect, $\alpha>1$ indicates a synergistic effect, and $\alpha<1$ indicates an antagonistic effect (Drewinko et al., 1976).

2.3. Detection of apoptosis/necrosis and cell cycle analysis

Apoptotic and necrotic cell death were analyzed by double staining with FITC-conjugated annexin V and propidium iodide (PI), in which annexin V binds to the apoptotic cells with exposed phosphatidylserine, while PI labels the necrotic cells with membrane damage. Staining was performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The green (FL1) and red (FL2) fluorescence of annexin/PI-stained cells was analyzed with FACSCalibur flow cytometer (BD, Heidelberg, Germany). The numbers of viable ($annexin^-/PI^-$), apoptotic ($annexin^+/PI^-$) and necrotic ($annexin^+/PI^+$) cells, were determined with a Cell Quest Pro software (BD). The cell cycle phases and DNA fragmentation, as another marker of apoptosis, were analyzed by flow cytometric measurement of red fluorescence (FL2) of DNA-bound PI in ethanol-fixed cells, exactly as previously described (Harhaji et al., 2007a,b).

2.4. Caspase activation

Activation of caspases was measured by flow cytometry after labeling the cells with a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (30 min at 37 °C). Cells were then trypsinized, washed with PBS to remove any unbound reagent and assayed by flow cytometry for detection of the bound reagent. The increase in green fluorescence (FL1) is a measure of caspase activity within individual cells of the treated population. The results are expressed as % of cells containing active caspases.

2.5. Oxidative stress measurement

Intracellular production of reactive oxygen species as an indicator of oxidative stress was determined by measuring the intensity of green fluorescence emitted by the redox-sensitive dye dihydrorhodamine 123 (DHR, Sigma). DHR (2 μM) was added to cell cultures at the beginning of treatment. At the end of incubation, cells were detached by trypsinization, washed in PBS, and the green fluorescence (FL1) of DHR-stained cells was analyzed using a FACSCalibur flow cytometer and a Cell Quest Pro software.

2.6. Mitochondrial depolarization

The mitochondrial depolarization was assessed using DePsipher (R&D Systems), a lipophilic cation (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl carbocyanin iodide) susceptible to the changes in mitochondrial membrane potential. It has the property of aggregating upon membrane polarization, forming an orange-red fluorescent compound. If the potential is disturbed, the dye can not access the transmembrane space and remains or reverts to its green monomeric form. The green monomer and the red aggregates were detected by flow cytometric analysis and the results were presented as a green/red fluorescence ratio (geomean FL1/FL2), the increase of which reflects mitochondrial depolarization.

2.7. Autophagy assessment

The apperance of acidic autophagic vesicles was detected by flow cytometry as previously described (Paglin et al., 2001).

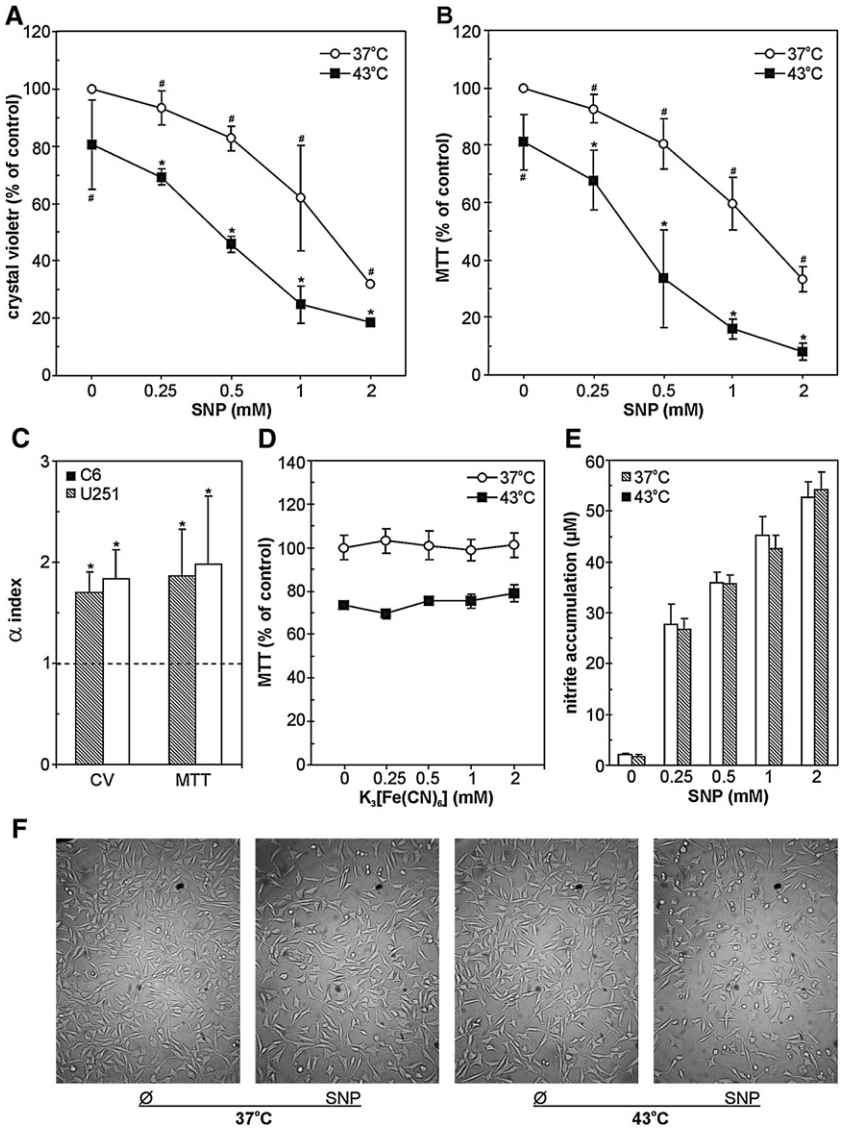

Fig. 1. Synergistic antiglioma effect of hyperthermia and SNP. C6 cells (A, C, D–F) or U251 cells (B, C) were treated with different concentrations of SNP (A, B, E), $K_3(Fe(CN)_6)$ (D) or 0.5 mM SNP (C, F), and incubated in normal (37 °C) or hyperthermic conditions (43 °C, 1 h). After 24 h, the cell viability was determined using crystal violet (CV) or MTT assay (A–D) and the α index for synergism/antagonism was calculated (C). Alternatively, the nitrite accumulation was measured in cell culture supernatants (E), or the cell morphology was examined by light microscopy (F). The results are presented as mean±S.D. values from three independent experiments (A–C) or mean±S.D. values of triplicate observations in one representative from at least three experiments with similar results (D, E) ($^{\#}P<0.05$ and $^{*}P<0.05$, respectively, refer to untreated cells and cell cultures with SNP only, while $^{*}P<0.05$ in C indicates values significantly higher than 1).

Briefly, after incubation the cells were trypsinized, washed, and incubated for 15 min at 37 °C with 1 μM acridin orange (Sigma). Acridine orange-stained cell nuclei are fluorescent green, while autophagic lysosomes are fluorescent orange-red. The increase in red vs. green ((FL3/FL1) fluorescence ratio, reflecting the autophagy, was determined using a FACSCalibur flow cytometer and a Cell Quest Pro software.

2.8. Nitrite determination

Nitrite accumulation, an indicator of nitric oxide production, was measured using the Griess reagent (Miljkovic et al., 2004). Briefly, 50 μl aliquots of cell culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) and incubated for 10 min at ambient temperature. The absorbance at 570 nm was measured in an automated microplate reader and nitrite concentration (μM) was calculated from a $NaNO_2$ standard curve.

2.9. Statistical analysis

The statistical significance of the observed differences was analyzed by *t*-test or ANOVA followed by the Student–Newman–Keuls test. The value of $P<0.05$ was considered significant.

3. Results

3.1. Synergistic antiglioma effect of hyperthermia and SNP

Both crystal violet and MTT assay demonstrated the ability of NO donor SNP to dose-dependently reduce the numbers of C6 and U251 glioma cells (Fig. 1A, B). The preliminary experiments have shown that exposure to hyperthermia (1 h at 43 °C) before SNP treatment was not able to modify its antiglioma effect (data not shown). On the other hand, a clear increase in SNP-induced glioma cell death was observed if hyperthermia was applied 1 h after the SNP treatment (Fig. 1A, B). While either SNP or hyperthermia reduced glioma cell numbers to a certain degree, the cytotoxicity of their combination exceeded the mere sum of the cytotoxic effects of each treatment alone, thus indicating a synergistic interaction (Fig. 1A, B). The synergistic cooperation of SNP and hyperthermia was further confirmed by mathematical analysis, showing that α values for combined treatment were significantly higher than 1 (Fig. 1C). In control experiments, $K_3(Fe(CN)_6)$ (potassium ferrycianide) in combination with hyperthermia failed to mimic the antiglioma effect of SNP (Fig. 1D), thus indicating that the ferrycyanide component of this NO donor was not involved in the observed effect. Moreover, the synergistic cytotoxicity was not simply due to the ability of hyperthermia to somehow augment SNP-mediated generation of NO, as we could not observe any difference in nitrite accumulation in control and hyperthermia-exposed cell cultures (Fig. 1E). Microscopic examination of C6 cells revealed that neither hyperthermia nor low dose (0.5 mM) of SNP alone was able to cause observable morphological changes (Fig. 1F). In contrast, some of the cells exposed to combined treatment lost their normal polygonal or spindle-like appearance, became smaller, rounded and eventually detached from cell culture plastic, which is consistent with the induction of cell death (Fig. 1F; similar results were obtained with U251 cells- data not shown).

3.2. Hyperthermia enhances the antiglioma effect of NO donors GSNO and PAPAnonoate

To further confirm the involvement of NO in the ability of SNP to cooperate with hyperthermia in killing glioma cells, we used GSNO and PAPA-NONOate, the NO donors with the chemical structures and NO-releasing mechanisms distinct from those of SNP. Similarly to SNP, both NO-releasing chemicals dose-dependently reduced the numbers of U251 glioma cells (Fig. 2A). Moreover, heating the cells for 1 h at 43 °C in the presence of

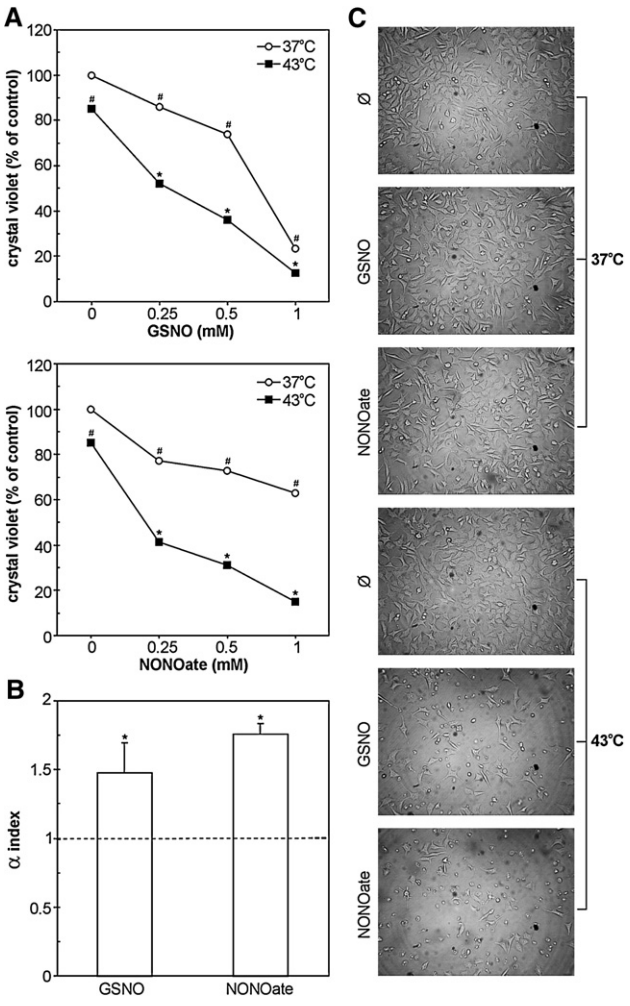

Fig. 2. Hyperthermia potentiates antiglioma effect of NO donors GSNO and PAPA-NONOate. U251 cells were incubated with different concentrations (A) or 0.25 mM (B, C) of GSNO or PAPA-NONOate in normal (37 °C) or hyperthermic conditions (43 °C, 1 h). Cell viability was determined by crystal violet staining 24 h after treatment (A, B), and the α index for synergistic/antagonistic interactions was calculated (B). The cell morphology was examined by light microscopy (C). The results from representative of three separate experiments were presented as mean±S.D. values of triplicates ($^{\#}P<0.05$ and $^{*}P<0.05$, respectively, refer to untreated cells and cell cultures with SNP only, while $^{*}P<0.05$ in B indicates values significantly higher than 1).

GSNO or PAPA-NONOate markedly potentiated their antiglioma effect (Fig. 2A), and the synergistic cooperation of hyperthermia and NO donors was further confirmed by the α values significantly exceeding 1 (Fig. 2B). While the morphology of U251 cells was not notably altered by either hyperthermia or NO donors at low concentrations (0.25 mM), after combined treatment the cells became smaller, rounded and detached from the well surface, indicating the loss of cell viability (Fig. 2C). Results similar to those presented in Fig. 2 were also obtained with C6 cells (data not shown). This ability of three different NO donors to display synergistic antiglioma activity in combination with hyperthermia suggest that NO was indeed involved in the observed effect.

3.3. Hyperthermia potentiates SNP-induced apoptosis and necrosis in glioma cells

We next examined the type of cell death induced by SNP and/or hyperthermia treatment, using double staining with annexin V-FITC and PI to detect phosphatydylserine exposure (annexin V) and cell membrane damage (PI) as the hallmarks of apoptosis and necrosis, respectively. Exposure of C6 glioma cells to hyperthermia or low dose of SNP (0.5 mM) did not induce significant cell death, while SNP at high concentration (2 mM) induced both apoptosis and necrosis (Fig. 3A). The combination of SNP and hyperthermia caused massive increase in number of apoptotic and necrotic cells in comparison with cell cultures treated with either SNP- or hyperthermia alone (Fig. 3A). The observed increase in number of necrotic cells was not due to a secondary necrosis following apoptotic cell death, as the time course experiments demonstrated an early (discernible after 12 h) appearance of PI^+ (necrotic) cells, which occurred simultaneously with phosphatydylserine exposure on PI^-(apoptotic) cells (data not shown). The DNA content analysis revealed that both SNP and hyperthermia led to an arrest in G_2M phase of the cell cycle (Fig. 3B). The combined treatment, however, did not further increase the G_2M cell cycle block, but caused a significant accumulation of cells in sub-G (hypodiploid) compartment, consistent with the fragmentation of DNA as one of the hallmarks of apoptotic cell death (Fig. 3B). The results similar to those presented in Fig. 3 were also obtained with U251 cells (data not shown). Therefore, the exposure to combination of hyperthermia and SNP leads to induction of both apoptotic and necrotic death in glioma cell cultures.

3.4. Hyperthermia and SNP synergize in inducing oxidative stress, mitochondrial depolarization and caspase activation in glioma cells

The oxidative stress and subsequent mitochondrial dysfunction are common death mechanisms in both necrosis and apoptosis, while the activation of aspartate-specific cysteine proteases belonging to caspase family is a crucial event in apoptosis execution. To examine the effect of hyperthermia and SNP on these

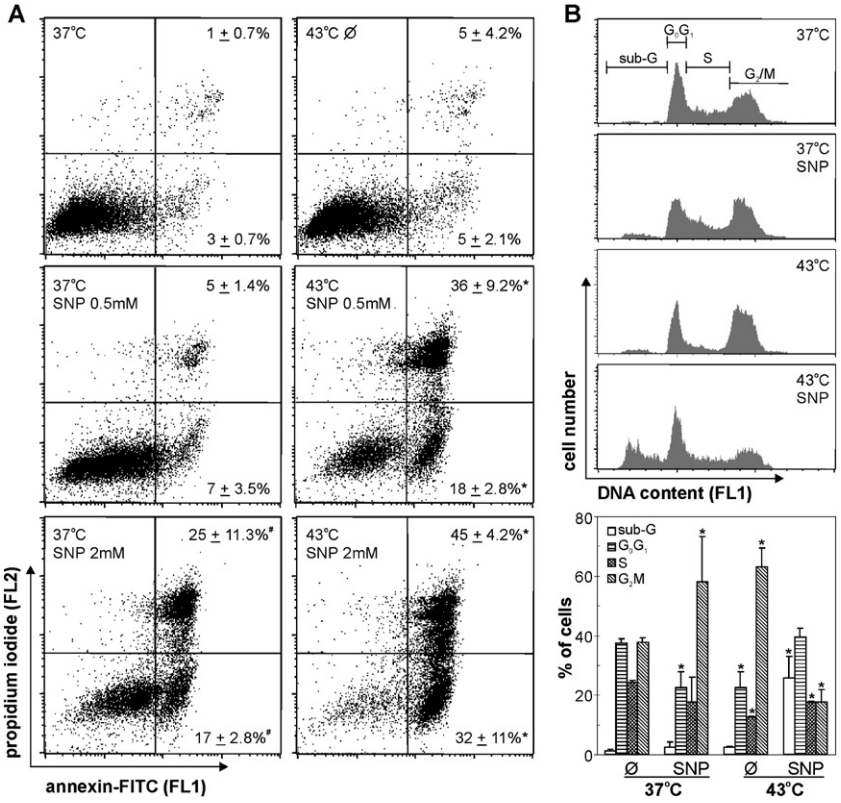

Fig. 3. Hyperthermia potentiates SNP-induced apoptosis and necrosis in glioma cells. C6 cells were treated with different doses (A), or 0.5 mM (B) of SNP, and incubated in normal (37 °C) or hyperthermic conditions (43 °C, 1 h). Apoptosis/necrosis (A) and cell cycle (B) were analyzed after 24 h using flow cytometry. The representative dot plots and histograms are presented, while the data in (A, B) are mean ± S.D. values from three independent experiments ($^{\#}P<0.05$ and $^{*}P<0.05$, respectively, refer to untreated cells and cells treated with SNP or hyperthermia alone).

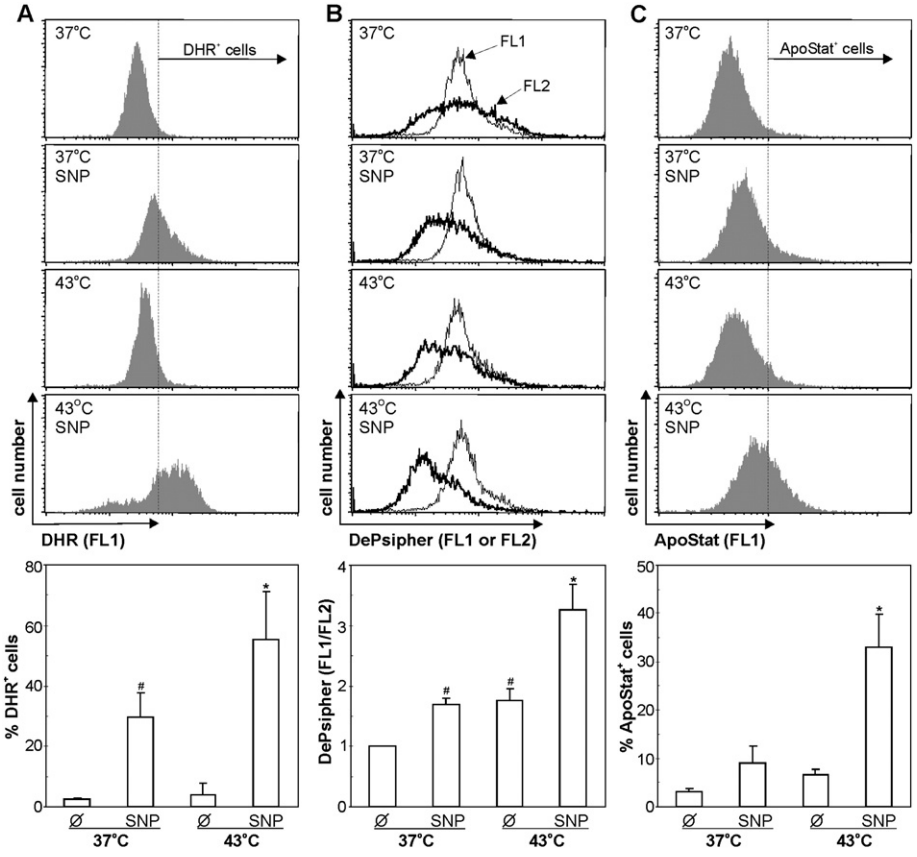

Fig. 4. Hyperthermia and SNP induce oxidative stress, mitochondrial depolarization and caspase activation in glioma cells. C6 cells were treated with SNP (0.5 mM) in normal (37 °C) or hyperthermic (43 °C, 1 h) conditions. Oxidative stress (DHR fluorescence; A), mitochondrial depolarization (DePsipher fluorescence; B) and caspase activation (ApoStat fluorescence; C) were analyzed by flow cytometry after 6 h (A) or 18 h (B, C). The representative histograms are presented, while the data shown in graphs are mean±S.D. values from three separate experiments ($^{\#}P<0.05$ and $^{*}P<0.05$, respectively, refer to untreated cells and cells treated with SNP or hyperthermia alone).

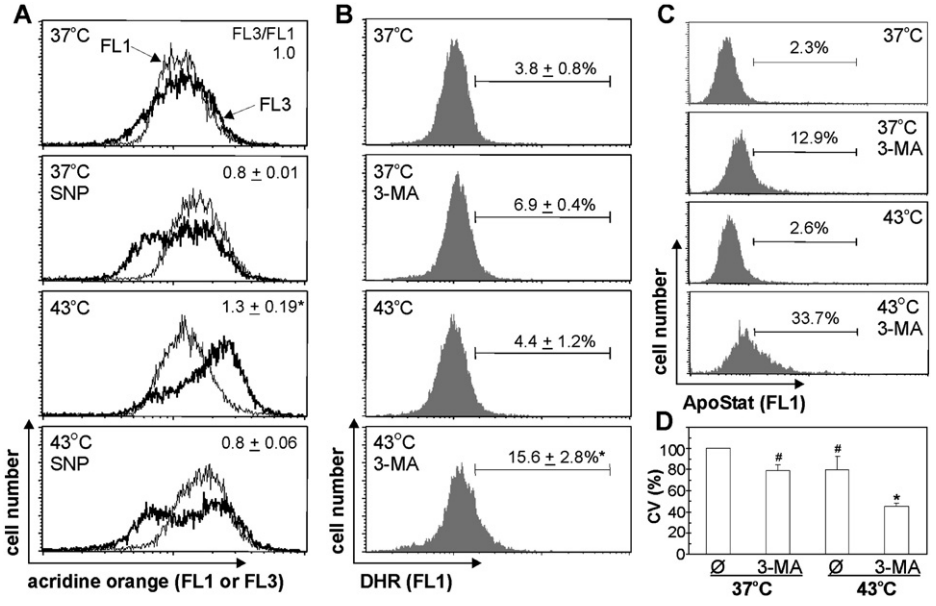

Fig. 5. The role of autophagy in hyperthermia+SNP induced glioma cell death. (A-D) C6 cells were treated with SNP (0.5 mM) in normal (37 °C) or hyperthermic (43 °C, 1 h) conditions. The presence of autophagic vesicles (acridine orange fluorescence; A), oxidative stress (DHR fluorescence; B) and caspase activation (ApoStat fluorescence; C) were analyzed by flow cytometry after 6 h (A, B) or 18 h (C). The cell viability was assessed by crystal violet (CV) assay after 24 h (D). The results in (A, B) are mean±S.D. values from three different experiments, while the data in (C) are mean values from one of two experiments with similar results, and the data in (D) are mean±S.D. values of triplicates from one of two experiments with similar results. ($P^{\#}<0.05$ and $P^{*}<0.05$, respectively, refer to untreated cells or all other treatments).

intracellular events, we used flow cytometric analysis of the cells stained with appropriate reporter dyes. While exposure to hyperthermia alone failed to induce a discernible oxidative stress, a moderate shift in green DHR fluorescence was evident in SNP-treated C6 cells (Fig. 4A). The most intense DHR fluorescence, however, was clearly observed after combined treatment with SNP and hyperthermia, suggesting a synergistic induction of oxidative stress (Fig. 4A). Both hyperthermia and SNP alone were able to cause mitochondrial depolarization to a certain degree, as judged by an increase in red/green fluorescence ratio of mitochondria-binding dye DePsipher (Fig. 4B). However, much more pronounced disruption of mitochondrial membrane potential was seen in cells treated with SNP and hyperthermia in combination (Fig. 4B). The staining with the fluorescent caspase inhibitor ApoStat revealed that neither hyperthermia nor SNP could induce statistically significant caspase activation in C6 cells, although a small increase in number of cells positive for caspase activity was observed following each treatment (Fig. 4C). On the other hand, a high proportion of glioma cells exposed to combined SNP/hyperthermia treatment displayed elevated ApoStat fluorescence, reflecting the synergistic activation of caspases (Fig. 4C). Results similar to those presented in Fig. 4 were also obtained with U251 cells (not shown). It therefore appears that the ability of hyperthermia and SNP to cooperate in the induction of glioma cell death is associated with the synergistic initiation of oxidative stress, mitochondrial depolarization and caspase activation.

3.5. The role of autophagy in hyperthermia + SNP-induced glioma cell death

As it has been reported that autophagic degradation of intracellular proteins occurs in hyperthermia-exposed glioma cells (Komata et al., 2004), we have investigated the possible role of autophagy in hyperthermia + SNP-induced glioma cell death. The ability of high temperature to induce autophagy in C6 cells was confirmed by staining with acridine orange, showing a characteristic increase in red (FL3) vs. green (FL1) fluorescence that signifies the appearance of acidified autophagic vesicles (Fig. 5A). On the other hand, SNP did not induce autophagy and actually prevented hyperthermia-mediated autophagy induction in glioma cells (Fig. 5A). The treatment with 3-methyladenine, a well-known inhibitor of autophagosome formation, led to a significant induction of oxidative stress (Fig. 5B), caspase activation (Fig. 5C) and cell death (Fig. 5D) in hyperthermia-exposed C6 cells ($\alpha = 1.4$ for combination of 3-methyladenine and hyperthermia in Fig. 5D). Therefore, the SNP-mediated inhibition of autophagy could at least partly contribute to a synergistic induction of glioma cell death in combination with hyperthermia.

4. Discussion

The present study for the first time demonstrates a synergistic in vitro antiglioma effect of hyperthermia and the NO donor SNP. Combined SNP/hyperthermia treatment caused both necrosis and caspase-dependent glioma cell apoptosis, possibly through synergistic induction of oxidative stress and mitochondrial depolarization that was partly enabled by SNP-mediated inhibition of protective autophagic response (summarized in Fig. 6). While recent studies revealed the ability of SNP to potentiate hyperthermia-mediated suppression of glioma growth in vivo (Krossnes et al., 1996, 1998), our data provide a novel insight into the mechanisms possibly underlying this beneficial action of SNP.

In accordance with the results of Komata et al. (2004), apoptosis/necrosis and cell cycle analysis in our study have shown that moderate antiglioma effects of hyperthermia were mainly due to a G_2M cell cycle arrest, rather than to an induction of glioma cell death. While SNP also caused a G_2M cell cycle block, it also displayed a dose-dependent ability to induce both apoptosis and necrosis in cultured glioma cell lines, as it has previously been described (Bernabe et al., 2001; Blackburn et al., 1998). The synergistic antiglioma effect of hyperthermia and SNP did not apparently stem from potentiation of cell cycle arrest, but primarily resulted from the increase in both apoptotic and necrotic glioma cell death. This could be particularly beneficial in anticancer therapy, as products released from necrotic cells have been shown to boost the host's immune responses against tumor (Johar et al., 2004). The failure of the ferricyanide moiety of SNP to collaborate with hyperthermia in killing glioma cells, as well as the ability of NO-releasing chemicals GSNO and PAPA-NONOate to mimic this SNP action, strongly indicate the NO-dependence of the synergistic antiglioma effect.

It has recently become apparent that mitochondrial dysfunction, manifested as a collapse of mitochondrial membrane potential, might be a common cellular reaction to various death signals, which, depending on cellular ATP level and other modifying factors, can culminate in apoptosis, necrosis, or the mixture of both (Denecker et al., 2001; Los et al., 2002; Lemasters, 1999). Indeed, the SNP + hyperthermia-mediated induction of glioma cell apoptosis and necrosis in our study has been preceded by synergistic increase in mitochondrial depolarization. This is consistent with the reported ability of NO to suppress mitochondrial respiration directly through inhibition of cytochrome oxidase (Brown, 1999), or indirectly via reaction with mitochondria-produced superoxide and formation of highly reactive peroxynitrite (Riobo et al., 2001). Furthermore, long-term hyperthermia (43 °C, 3h) has been shown to cause mitochondrial dysfunction

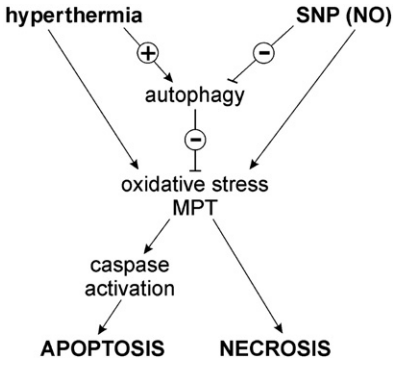

Fig. 6. Schematic representation of the mechanisms underlying synergistic antiglioma effect of hyperthermia and SNP (MPT-mitochondrial permeability transition).

and subsequent production of large amounts of reactive oxygen species by the electron transfer chain (Flanagan et al., 1998; Qian et al., 2004). Accordingly, we have observed that mitochondrial depolarization triggered by combination of SNP and hyperthermia was accompanied by synergistic increase in generation of reactive oxygen intermediates, thus providing a positive feedback mechanism leading to further mitochondrial injury and oxidative stress-dependent cell demise. The loss of inner mitochondrial membrane potential is associated with mitochondrial swelling and rupture of the outer mitochondrial membrane. During this process, called mitochondrial permeability transition (Tsujimoto and Shimizu, 2007), the inner mitochondrial membrane becomes freely permeable to protons, causing uncoupling of oxidative phosphorylation and necrosis due to ATP depletion (Bal-Price and Brown, 2000; Brown and Borutaite, 2002). In the presence of sufficient amount of glycolytic ATP, however, an apoptotic process will be triggered through mitochondrial permeability transition-dependent release of small molecules such as cytochrome c and subsequent activation of caspase cascades (Loeffler and Kroemer, 2000; Ferrari et al., 1998). The activation of caspases by SNP+hyperthermia has been demonstrated in our study, thus further confirming that both necrosis and caspase-dependent apoptosis are simultaneously initiated in glioma cells exposed to combination of these cytotoxic stimuli.

Our unpublished data confirm earlier results that longer exposure (2–3 h) to 43 °C, unlike short-term hyperthermia, can trigger apoptotic death of glioma cells (Janjetovic et al., unpublished). Moreover, the results of previous and the present report suggest that NO and hyperthermia employ similar cytotoxic mechanisms, including induction of mitochondrial depolarization, oxidative stress and caspase activation (Fig. 6). With this in mind, a question arises as to the mechanisms possibly underlying the synergistic antiglioma effect of the two treatments. Autophagy, a process of self-cannibalization responsible for the removal of long-lived proteins and damaged organelles through a lysosomal degradation pathway, has recently been recognized for its ability to increase or block tumor cell killing in a context-dependent manner (Bauvy et al., 2001; Bursch et al., 2000; Harhaji et al., 2007a,b; Kanzawa et al., 2003, 2004). In accordance with the previous report (Komata et al., 2004), we have demonstrated the appearance of autophagic vesicles in glioma cells exposed to hyperthermia. SNP, on the other hand, did not induce autophagy in our experiments, which is consistent with recent microarray analysis, showing inability of NO to induce autophagy-related genes (Rabkin and Klassen, 2007). However, SNP completely downregulated hyperthermia-induced autophagy in glioma cells, while inhibition of autophagy with 3-methyladenine mimicked the ability of SNP to trigger oxidative stress, caspase activation and apoptosis in glioma cells exposed to hyperthermia. The latter result is consistent with the increase in glioma cell apoptosis observed when autophagy was inhibited during hyperthermic treatment (Komata et al., 2004). It should be noted, however, that Komata et al. (2004) described this effect using a proton pump blocker bafilomycin A1, while autophagosome formation inhibitor 3-methyladenine was inactive in their hands. This discrepancy, however, might be explained by the fact that we have used 3-methyladenine at 10 mM, which is an order of magnitude higher dose compared to that employed in the previous study (Komata et al., 2004). Nevertheless, the dosage of 3-methyladenine in our experiments was not unusually high, as the same concentration (10 mM) has been routinely used by other researchers to efficiently prevent autophagy (Espert et al., 2006; Herman-Antosiewicz et al., 2006). Our data indicate that the synergistic antiglioma effect of nitric oxide and hyperthermia was at least partly enabled by SNP-mediated suppression of cytoprotective autophagic response (Fig. 6). This hypothesis is consistent with the absence of synergistic killing when the sequence of cytotoxic stimuli was reversed (SNP after hyperthermia), having in mind that the late addition of SNP was probably not able to suppress autophagy that was already induced by hyperthermic treatment. While the mechanisms underlying the NO-mediated suppression of autophagy remain to be explored, they could possibly involve nitrosylation of cellular proteins involved in autophagic process. By revealing a possible role of autophagy block in hyperthermia+nitric oxide-mediated antiglioma effect, our data further support the concept that tumor cells in some conditions might employ autophagy as a mechanism to evade therapy-induced death.

In conclusion, the present study clearly demonstrates a synergistic cooperation of hyperthermia and NO donor SNP in inducing mitochondrial depolarization, oxidative stress and subsequent apoptotic/necrotic death in glioma cells. Interestingly, it has previously been described that hyperthermia can actually protect normal primary astrocytes from oxidative stress-induced necrosis (Thomas et al., 2002), thus providing grounds for development of relatively non-toxic, tumor cell-targeted therapeutic approach. Having in mind the promising results of recent clinical trials in which hyperthermic treatment of malignant gliomas was well tolerated and showed some effectiveness (Fiorentini et al., 2006; Maier-Hauff et al., 2007; Sneed et al., 1998), as well as the fact that SNP is a clinically approved drug, a combined hyperthermia/nitric oxide treatment seems worthy of further investigation in glioma therapy.

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