Effect of Peroxynitrite on Production of Superoxide Anion Radicals and Calcium Homeostasis in Cells of Astroglial Origin

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We studied the effect of a donor of peroxynitrite, SIN-1, on the morphological characteristics of interweaved rat C6 glioma cells, on menadione-induced production of superoxide anion radicals, and on the concentration of Ca^{2+} in these cells. In concentrations of $1.25 \cdot 10^{-4}$ to $2.5 \cdot 10^{-7}$ M, SIN-1 demonstrated cytotoxic and antimitogenic effects. This donor of peroxynitrite caused abnormal modifications of the size of C6 cells and the structure of cellular organelles, intensified in a dose-dependent manner the release of Ca^{2+} from cellular stores into the cytoplasm, and suppressed menadione-induced production of superoxide anion radicals. Therefore, it should be believed that peroxynitrite exerts a modifying effect on the processes of mitotic division and induces apoptosis; it is also involved in the processes of intracellular signalling providing an increase in the concentration of cytosolic Ca^{2+} and a decrease in the redox activity of cells.

Keywords: interweaved C6 glioma cells of rat, peroxynitrite, menadione-dependent chemiluminescence, cytoplasmic concentration of Ca^{2+}.

INTRODUCTION

Astrocytes, one of the main types of glial cells of the brain, fulfill a number of important functions in the CNS. They are involved in the control of extracellular concentration of ions, metabolites, and neurotransmitters [1], as well as in the processes of neurogenesis, synaptogenesis, vasculogenesis, angiogenesis, remyelinization, etc. [2]. Together with cerebral endothelial cells, astrocytes form the blood-brain barrier [3]; they are involved in the initiation and control of immune and inflammatory responses to damage and in the formation of a number of pathological states of the brain [4].

It is known that the action of a peroxynitrite anion ($ONOO^-$) induces single-filament disruption between molecular chains of DNA, intensifies peroxidation of membrane lipids and lipoproteins, induces modifications of amino acid residues (components of protein molecules), and inhibits the process of mitochondrial respiration [5, 6]. These changes can result in significant modifications of the morphological characteristics and functional activity of cerebral cells. Such modifications are important pathogenetic factors initiating or intensifying neurodegenerative processes (in postinsult states, multiple sclerosis, Parkinson's disease, etc.) [7].

It was demonstrated that peroxynitrite decreases the level of reduced glutathione and mitochondrial transmembrane potential in cerebral cells under conditions of glucose deficiency [8]. In primary astrocytes and interweaved C6 glioma cells of the rat (i.e., cells of the astrocyte origin) stimulated by lipopolysaccharide B and interferon γ, the action of a peroxynitrite donor, SIN-1 ($1 \cdot 10^{-8}$ to $1 \cdot 10^{-9}$ M), results in a drop in the concentration of intracellular ATP [9]. Through specialized transport proteins, $ONOO^-$ induces the release from astrocytes of L-arginine, which is involved in the synthesis of nitric oxide in neurons [10]. Upon the action of micromolar concentrations of SIN-1, transformation of glucose

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via a pentose phosphate pathway in astrocytes is intensified; as a result, NADP·H is accumulated in the cells [11]. Incubation of primary astrocytes and cells of the C6 strain with SIN-1 in millimolar concentrations is accompanied by the release of lactate dehydrogenase into the extracellular milieu, which is indicative of a disturbance of the integrity of the cell membranes [12].

Peroxynitrite is produced in the CNS in endothelial cells of the vessels, microglial cells activated by cytokines and β-amyloid peptide, and neurons (in the case where there is a deficiency of L-arginine or tetrahydrobiopterin) [7]. It was shown that astrocytes produce superoxide anion radicals upon the action of arachidonic acid and anaphylatoxin of the system of complement C5a [13, 14]. As a result of interaction between these radicals and nitric oxide, peroxynitrite is produced [5].

Many studies were aimed at examination of the effects of peroxynitrite on morphological and functional properties of astroglial cells; however, the mechanisms underlying the action of this intermediate remain unstudied in detail. To elucidate the mechanisms of the action of $ONOO^-$ on astroglial cells, we examined the effect of its donor, SIN-1, on morphological characteristics of interweaved C6 glioma cells of the rat, as well as on the menadione-dependent production of superoxide anion radicals by these cells and on the intracellular concentration of calcium ions.

METHODS

The strain of interweaved C6 glioma cells of the rat was obtained from the cell culture collected at the Institute of Cytology of the Russian Academy of Sciences (Saint Petersburg, Russia). The cells were cultured in Eagle MEM medium (Sigma, USA) with the addition of 10% serum of cow fetuses (Institute of Epidemiology and Microbiology of the Ministry of Public Health, Republic of Belarus) and $1\cdot10^{-4}$ g/ml gentamycin (Belmedpreparaty, Republic of Belarus) in flat flasks and on cover glasses put in such flasks. The plating concentration of the cells was $1.5\cdot10^5$ ml^{-1} [15]. After 48 h of culturing (within a logarithmic phase of growth), the growing medium was substituted for the maintaining one (Eagle MEM medium with no serum). Then, solutions of a donor of peroxynitrite, SIN-1 (3-morpholinosydnonimine-N-ethyl carbamide, Sigma, USA) at final concentrations of $1.25\cdot10^{-4}$ to $2.5\cdot10^{-7}$ M were introduced in flasks for 24 h. The volume of the added solution did not exceed 10% of the total volume of the sampling. The obtained cell preparations were fixed in the Dubosq–Brasil–Bouin liquid, stained with hematoxylin/eosin (Sigma, USA), and then analyzed using a light microscope (magnification 1,000×).

Menadione-dependent generation of superoxide anion radicals in the suspension of C6 cells was recorded with the help of a chemiluminescence technique using a biochemiluminometer at 37°C and pH 7.4. Lucigenin (10,10-dimethyl-biacridine, Fluka, Switzerland) in a $1.25\cdot10^{-5}$ M concentration was used as an emitter of luminescence. Prior to the measurement, the cells fixed to the flask surface were removed using Versene (Analysis-X, Republic of Belarus). The suspension obtained was washed off with Earle's balanced salt solution (EBSS) of the following composition (M): NaCl, 0.12; KCl, $5.4\cdot10^{-3}$; $NaHPO_4\cdot2H_2O$, $0.9\cdot10^{-3}$; $MgSO_4\cdot7H_2O$, $0.8\cdot10^{-3}$; glucose, $5.6\cdot10^{-3}$; $CaCl_2$, $1.8\cdot10^{-3}$; $NaHCO_3$, $2.62\cdot10^{-2}$ (ANAliz-X, Republic of Belarus). The concentration of the cells was adjusted to $1\cdot10^6$ ml^{-1} with EBSS. Menadione (2-methyl-1,4-naphthoquinone; Sigma, USA) was used in the concentration of $1.62\cdot10^{-5}$ M.

The level of inhibition of the integral intensity of lucigenin-mediated menadione-dependent chemiluminescence (MDCL) of the suspension of C6 cells (ΔS) was calculated by the following formula:

$$\Delta S = 100\% - (\Sigma I_{SIN\text{-}1}/\Sigma I_K)\cdot100\%,$$

where $\Sigma I_{SIN\text{-}1}$ is the integral intensity of lucigenin-mediated MDCL of the cells upon the action of SIN-1 applied in different concentrations, and ΣI_K is the integral intensity of MDCL of the C6 cells in the control sampling.

The concentration of free calcium ions in the cytoplasm ($[Ca^{2+}]_{cyt}$) was estimated using a fluorescence technique on a spectrofluorimeter; a calcium-sensitive probe, Fura PE-3AM (Sigma, USA), was used [16]. The C6 cells were incubated in the EBSS for 1 h at 37ºC with Fura PE-3AM in the concentration of $2\cdot10^{-6}$ M; then the suspension was washed twice, adjusted to the concentration of $2\cdot10^6$ ml^{-1} with BESS, and again incubated for 30 min in a thermostat. The measurements were performed at 37ºC and pH 7.4. Prior to the measurements, SIN-1 in different concentrations was added to the analyzed samplings.

The viability of cells was estimated on a spectrofluorimeter, using also a fluorescence technique with propidium iodide [17], whose molecules enter the cells only in the case where the cytoplasmic membranes

are damaged; these molecules are incorporated between DNA and RNA nucleotides. After culturing of the samplings of C6 cells in the presence of SIN-1 for 24 h, a monolayer of these cells was washed off twice with EBSS. After this, the propidium iodide solution ($4 \cdot 10^{-6}$ M) was added, and, after mixing, we measured the intensity of fluorescence F_{d1} ($\lambda_{excitation}$ = = 530 nm, λ_{rec} = 645 nm) on the 5th min. Then, the cells were destroyed with a detergent, Triton X-100, and the intensity of fluorescence F_{t1} was recorded. The viability of the cells was estimated using the formula

$$\frac{(F_{t1} - F_{t2}) - (F_{d1} - F_{d2})}{(F_{t1} - F_{t2})} \cdot 100\ \%,$$

where F_{d2} and F_{t2} are the intensities of fluorescence of propidium iodide and Triton X-100, respectively, in the EBSS.

The obtained numerical data are presented as means ± ± s.d.

RESULTS AND DISCUSSION

Cells of the C6 strain are units of astrocyte origin. Although they differ from primary astrocytes in some characteristics, the correctness of transfer of the main data obtained in the experiments with this cell strain on "normal" astrocytes is beyond question.

It was demonstrated that $1 \cdot 10^{-4}$ M SIN-1 in phosphate buffer at pH 7.4 and 37ºC produces NO and O_2^-, and, due to interaction between these agents, peroxynitrite is produced [18]. In the course of culturing of interweaved C6 glioma cells of the rat in the presence of SIN-1 in concentrations from $1.25 \cdot 10^{-4}$ to $2.5 \cdot 10^{-7}$ M for 24 h, we found rather clear changes in the morphological characteristics of the cells of this strain, which are indicative of the cytotoxic action of peroxynitrite. In the studied samplings, we observed shortening and thickening of the cell processes, vacuolization of the cytoplasm, changes in the dimensions of cells and their nuclei, and pycnosis of the nuclei.

All changes of the morphological characteristics of C6 cells developed in a local manner, i.e., they were not observed in all sites of the sampling. The level of manifestation of these modifications in samplings of cultured C6 cells depended on the concentration of SIN-1. In the case where the concentration of this agent was higher than $1.25 \cdot 10^{-4}$ M, we observed damage to the integrity of the monolayer, rounding off of the cells, and destruction of their processes, i.e., peroxynitrite exerted a clear cytodestructive effect. These results agree well with the published data that, upon the action of $ONOO^-$ in millimolar concentrations, the monolayer of the C6 cells rather rapidly (in 40 min) becomes mostly separated from the surface of the culturing flask, and, in 24 h, the release of lactate dehydrogenase is equal to 95% [12].

Upon the action of SIN-1 applied in micromolar concentrations to C6 cells, we first observed pathological (clearly deviating from the norm) phases of mitosis. As a result, conglomerates of polynuclear cells, with superposition of the nuclei on each other, were formed [19]. Therefore, peroxynitrite modifies the mitotic division of C6 cells. In the course of mitosis, the cell exercises self-control of its own state. When excessive (nonreparable or poorly reparable) damages to the chromosomes develop (numerous disruptions of DNA, disturbances of its conformation, "sewings together" between chains, and/or incorrect segregation of chromosomes), as well as serious disturbances of the cellular membranes (especially, mitochondrial ones) related to lipid peroxidation appear, the mechanism underlying apoptosis is triggered [20]. Since $ONOO^-$ initiates nearly all the above-listed pathological changes in the cells, it can play the role of an inductor of apoptosis. This process triggered by the action of peroxynitrite on cells of different types was described earlier [21, 22]. When C6 cells were cultured together with $(0.5 \text{ to } 0.25) \cdot 10^{-6}$ M SIN-1 for 24 h, we observed single cells with manifestations of apoptosis. In the analyzed samplings, the following morphological modifications were observed: fragmentation of the nuclei and cytoplasm with formation of apoptotic bodies, wrinkling of the cells, pycnosis of the nuclei, the appearance of conglomerates of chromatin adjacent to the membrane, and condensation and vacuolization of the cytoplasm [19].

To quantitatively estimate the effect of the peroxynitrite donor on the morphological characteristics of cultured C6 cells (24-h-long culturing), we tested the viability of the cells using propidium iodide. In this case, we used the concentration of SIN-1 that provided preservation of the cell monolayer. As can be seen in Fig. 1, with decrease in the concentration of SIN-1 from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-5}$ M, the relative number of intact (living) cells sharply increased (up to 80%). With further decreases in the concentration of the above agent, the above parameter remained constant (within an error range) but did not reach the level observed in control samplings. Based on the results of microscopic study and taking into account the facts that propidium iodide selectively stains apoptotic and necrotic cells [17], while peroxynitrite, according

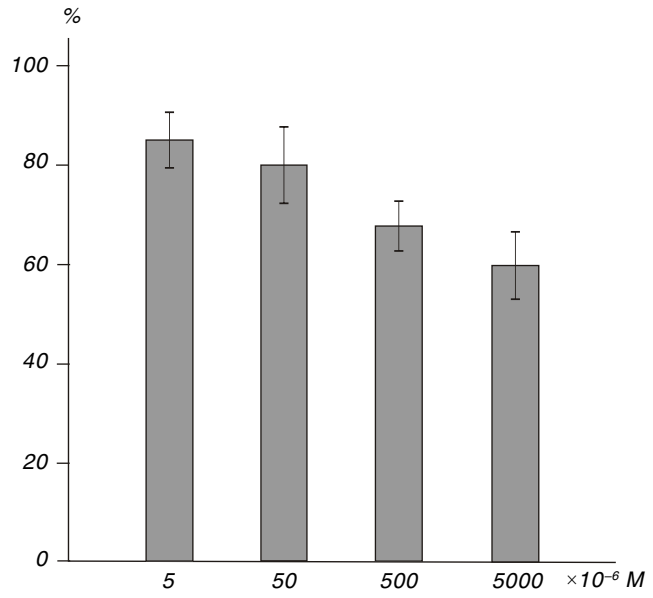

Fig. 1. Index of viability (%) of interweaved C6 glioma cells upon the action of a donor of peroxynitrite, SIN-1, applied for 24 h in different concentrations. The viability of cells in control samplings (24-h-long culturing in a SIN-1-free medium) is taken as 100%.

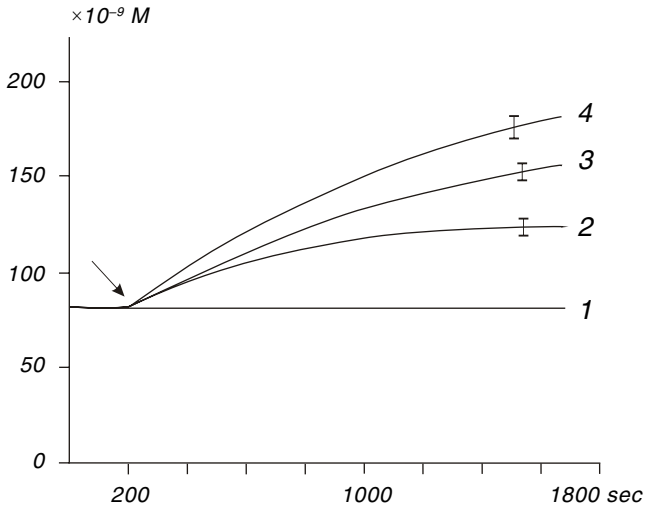

Fig. 2. Kinetic dependence of changes in the concentration of free (ionized) cytosolic calcium ($[Ca^{2+}]_{cyt}$) in interweaved rat C6 glioma cells upon the action of SIN-1. 1) Control ($[Ca^{2+}]_{cyt}$ in the absence of SIN-1); 2-4) at SIN-1 concentrations of $5 \cdot 10^{-6}$ (2), $5 \cdot 10^{-5}$ (3), and $5 \cdot 10^{-4}$ (4) M.

to the published data [6, 7, 21, 22], induces both necrotic and apoptotic modifications, we believe that the presence of a noticeable number of damaged cells in the system under examination upon the action of SIN-1 applied even in rather small concentrations is determined by cell death triggered by one of the above mechanisms.

It is known that changes in the concentration of calcium ions are one of the main processes providing intracellular signalling [23, 24]. Upon the action of many (but not all) inductors of apoptosis, an increase in the $[Ca^{2+}]_{cyt}$ related mostly to the activation of Ca-/Mg-dependent endonuclease and Ca-dependent proteinase of calpain [20, 21] is observed. We studied the effect of peroxynitrite on the level of intracellular Ca^{2+} in rat C6 glioma cells. In Fig. 2, the dependences of $[Ca^{2+}]_{cyt}$ on the duration of culturing of C6 cells in the presence of SIN-1 in different concentrations are shown. It can be seen that SIN-1 induced a rise in the cytosolic concentration of Ca^{2+} in a dose-dependent manner. After addition of this agent in concentrations higher than $5 \cdot 10^{-5}$ M to the cell suspension, we observed a slow increase in the $[Ca^{2+}]_{cyt}$. In the case where SIN-1 was applied in lower doses, a relatively moderate rise (no more than by 50%) first occurred, and then the level of $[Ca^{2+}]_{cyt}$ remained constant over a long period.

Recently, we demonstrated that the ability of the rat interweaved C6 glioma cells to produce superoxide anion radicals upon the action of menadione reflects the functional state of these astrocyte-derived cells [25, 26]. Changes in the mitotic activity of C6 cells upon the action of lypopolysaccharide B used in different concentrations positively correlated with menadione-dependent production of $O_2^{\bullet -}$ [25]. We used this technique for further studying the antimitotic effect of SIN-1. Figure 3 illustrates typical kinetic dependences of lucigenin-mediated MDCL of the rat interweaved C6 glioma cells. Menadione applied in the concentration of $1.6 \cdot 10^{-5}$ M caused a typical chemiluminescence reaction of C6 cells, including two components, a fast phase (5 to 15 min) and a slow phase (>15 min). It can be seen that, from the first minutes after the addition of SIN-1 to the cell suspension, the intensity of both phases of MDCL decreased (Fig. 3, curves 1 and 2). The observed effect depended on the peroxynitrite concentration. Figure 4 illustrates the dependence of the level of inhibition of the integral intensity of lucigenin-mediated MDCL on the SIN-1 concentration. Upon the action of $1 \cdot 10^{-7}$ M SIN-1, the inhibitory effect of this donor was insignificant (7%). With further rise in the SIN-1 concentration, the level of inhibition increased. Suppression of MDCL of C6 cells induced by SIN-1can be considered an additional confirmation of the antimitotic effect of peroxynitrite, since the above test, as was demonstrated earlier, is an independent indicator of the mitotic activity in this cell culture [25, 26].

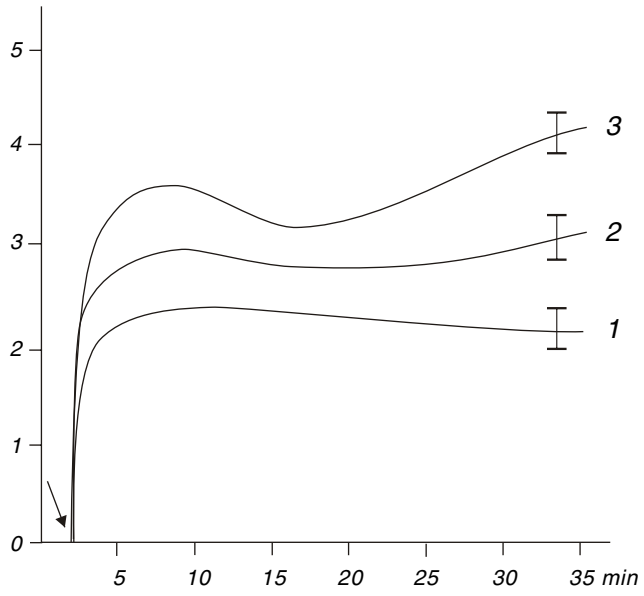

Fig. 3. Kinetic dependence of the intensity of menadione-dependent lucigenin-mediated chemiluminescence of the suspension of interweaved rat C6 glioma cells in the control (3) and after addition of a donor of peroxynitrite, SIN-1, in concentrations of $12.5 \cdot 10^{-6}$ (1) and $5 \cdot 10^{-7}$ (2) M. The concentrations of menadione and lucigenin were, respectively, $1.6 \cdot 10^{-5}$ and $12.5 \cdot 10^{-6}$ M. Arrow indicates the moment of addition of menadione.

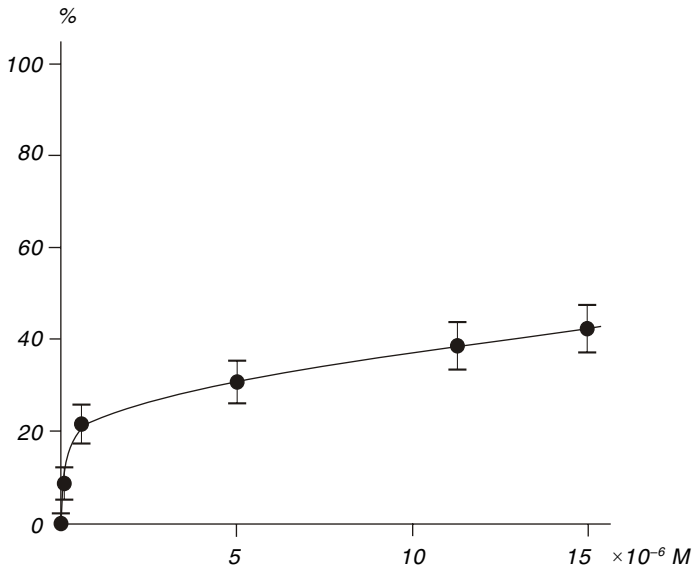

Fig. 4. Dependence of the level of inhibition of the integral intensity of menadione-dependent lucigenin-mediated chemiluminescence (ΔS, ordinate) of the suspension of C6 cells on the concentration of SIN-1 (abscissa, 10^{-6} M). The concentrations of menadione and lucigenin were, respectively, $1.6 \cdot 10^{-5}$ and $12.5 \cdot 10^{-6}$ M.

Therefore, our data indicate that the morphological characteristics and functional properties of the interweaved rat C6 glioma cells undergo significant modifications upon the action of SIN-1 used within a $1.25 \cdot 10^{-4}$ to $2.5 \cdot 10^{-7}$ M concentration range. Peroxynitrite induced abnormal changes in the size and structure of cellular organelles of the studied astroglia-derived cells and also exerted a modifying effect on the processes of mitotic division; this resulted in the induction of apoptosis. Toxic and antimitogenic effects of SIN-1 were manifested already within the initial stage of interaction between this agent and C6 cells. We hypothesize that peroxynitrite is involved in the processes of intracellular signalling because the cytosolic concentration of Ca^{2+} increases and the redox activity of the cells drops upon the action of this agent.

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