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Peroxynitrite Causes Aspartate Release from Dissociated Rat Cerebellar Granule Neurones

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Peroxynitrite ($ONOO^-$) is a powerful oxidant which is formed from the reaction between nitric oxide (NO) and superoxide anion. It has therefore been proposed to mediate the toxic actions caused by NO. Since $ONOO^-$ may be formed in the central nervous system (CNS) in pathological conditions such as brain ischaemia, we decided to investigate whether this molecule induces the release of the endogenous excitatory amino acids glutamate and aspartate from neurones. We selected as biological model acutely dissociated rat cerebellar granule neurones in suspension to allow a direct interaction between $ONOO^-$ and target cells. Peroxynitrite caused a concentration-dependent release of aspartate but not of glutamate from dissociated cerebellar granule neurones. Peroxynitriteinduced aspartate release was inhibited by dithiothreitol, tetrodotoxin, and in Na^+-deprived solutions and not affected by EGTA or pre-incubation with the cytosolic Ca^{2+} chelator BAPTA/ AM. Peroxynitrite also induced an increase in intracellular Ca^{2+} concentration which was not affected in the presence of EGTA. These data show that $ONOO^-$ causes release of aspartate from cerebellar granule neurones and that this effect might arise from an alteration of Na^+ membrane permeability leading subsequently to reversal of a Na^+-dependent plasma membrane transporter of this excitatory amino acid. In addition, $ONOO^-$ alters Ca^{2+} homeostasis likely due to Na^+ overload. Taken together, these findings may help and elucidate some of the intimate mechanisms of NO-induced neuronal damage in pathological circumstances.

Keywords: Excitatory amino acids, intracellular Ca^{2+}, nitric oxide, Na^+ transport, peroxynitrite, dissociated cerebellar granule neurones

Abbreviations: NO: Nitric oxide; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EAA: excitatory amino acids; EGTA: ethylene glycol-bis(β-aminoethylether) *N,N,N′,N′*-tetraacetic acid, TTX: tetrodotoxin, DTT: dithiothreitol; BAPTA/AM: [1,2-bis-(o-Aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester]; DHK: dihydrokainic acid; Fura 2/AM: 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]- 2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid pentaacetoxymethylester; BSA: bovine serum albumin; DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid); SNAP: S-nitroso-*N*-acetyl-DL-penicillamine; CNS: central nervous system; GABA: γ-aminobutyric acid.

INTRODUCTION

Although nitric oxide (NO) plays several physiological roles, its excessive production may have

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cytotoxic effects.[1] Part of the toxicity of NO involves its reaction with superoxide radical (O_2^-) to form the powerful oxidant peroxynitrite ($ONOO^-$).[2] Peroxynitrite has been reported to have strong oxidising properties,[3–5] to react with transition metals to form a powerful nitrating agent,[6] and to react with DNA[7,8] and sugars.[3,9–11] Because its formation has been reported to occur *in vivo*,[12] $ONOO^-$ might be synthesised in the CNS when both NO and O_2^- are formed at high concentrations, as it is the case in brain ischaemia.[13,14] Indeed, $ONOO^-$ has been shown to cause neuronal death[15,16] and it has been proposed to occur in different disorders of the CNS such as brain ischaemia, AIDS-associated dementia, amyothrophic lateral sclerosis, etc.[17]

On the other hand, aspartate and glutamate are the major excitatory neurotransmitters in the mammalian brain where they serve important physiological functions, such as participation in fast excitatory synaptic transmission and modulation of synaptic plasticity. However, they are able to cause neuronal death[18] and, since the first report showing that extracellular concentrations of glutamate and aspartate increased in the rat hippocampus during ischaemia,[19] numerous studies have confirmed and characterised this phenomenon in several brain regions and with different models of ischaemia.[20] Moreover, their potent neuroexcitatory effects are involved in cellular death in a wide range of neurological disorders.[21,22]

Since it has been reported that $ONOO^-$ causes the release of neurotransmitters such as acetylcholine and γ-aminobutyric acid (GABA) from cortical neurones,[23,24] we decided to examine the possibility that $ONOO^-$ may induce excitatory amino acid release by investigating the effects of $ONOO^-$ on the efflux of endogenous aspartate and glutamate from neuronal cells. We selected as biological model acutely dissociated rat cerebellar granule neurones in suspension to allow a direct interaction between $ONOO^-$ and the target cells. Some of these data have been presented in a preliminary form elsewhere.[25]

MATERIALS AND METHODS

Isolation of Rat Cerebellar Granule Cells

Cell suspensions were prepared as described.[26,27] Briefly, cell suspensions were obtained from young (6–8 days postnatal) Wistar rat cerebella by mild trypsin treatment followed by trituration and were resuspended in a solution (incubation solution) containing (mM): 130 NaCl, 3 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 15 Tris-HCl, and 11 glucose (pH 7.4 at 37°C).

Excitatory Amino Acid Release Experiments in Cell Suspensions

Peroxynitrite (100–500 μM) or decomposed $ONOO^-$ was added to cerebellar granule neurones (10^7 cells/ml, 0.5 ml) resuspended in incubation solution at 37°C with continuous stirring. In some experiments, the effects of the Ca^{2+} chelator EGTA (1 mM), the Na^+ channel blocker tetrodotoxin (TTX, 1 μM; added 10s after the addition of $ONOO^-$), the disulphide reducing agent dithiothreitol (DTT; 1 mM; added 10s after the addition of $ONOO^-$), the EAA uptake inhibitor dihydrokainic acid (DHK, 10 mM) and the intracellular Ca^{2+} chelator BAPTA/AM (10 μM) on $ONOO^-$ (400 μM)-induced aspartate release were studied. When EGTA was tested, incubation solution with no added $CaCl_2$ was used. For the experiments with BAPTA/AM, cells were loaded in the presence of this compound (10 μM) for 45 min at 37°C. Some agents (as indicated) were added 10 s after $ONOO^-$ to avoid a direct reaction with this molecule. In another set of experiments, NaCl in the incubation solution was substituted equiosmotically by choline, and the effects of 400 μM $ONOO^-$ were studied. For all the treatments, 1 min after the addition of $ONOO^-$, 4.5 ml of incubation solution were added and supernatant was collected for the determination of the excitatory amino acids (EAA) aspartate and glutamate after centrifugation at 200×g for 10 min. Some additional

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sets of control experiments were performed in cells not treated with $ONOO^-$: to test the ability of the cells to release EAA, cells were challenged with depolarising concentrations of KCl (50 mM); to examine the effectiveness of the Ca^{2+} chelators EGTA and BAPTA/AM, their effects were studied on KCl (50 mM)-induced EAA release; to explore the effects of NO on excitatory amino acid release from this preparation, some cells were incubated with the S-nitrosothiol S-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 30 μM). In all these cases, supernatant was collected for EAA analysis as described above. Data are expressed either as ng of total EAA release/10^7 cells or, when indicated, as net EAA release/10^7 cells (ng EAA after subtracting the basal release).

HPLC Determination of EAA Concentration

Analysis of EAA in each sample was performed by HPLC with fluorimetric detection (Perkin Elmer Binary LC Pump 250 and Fluorescence Detector LC 240) following precolumn derivatisation with the o-phtalaldialdehyde procedure.[28] EAA derivatives were separated isocratically on a reverse phase column (4.6×150 mm, 5 μm particle diameter, Nucleosil 100-C18) using a mobile phase consisting of sodium acetate buffer (0.05 M pH6.5), 20% methanol and 2% tetrahydrofuran. The area of each peak was determined with a Perkin Elmer Nelson Model 1020 integrator (Phoenix 8088 ROM BIOS Version 2.52 software), and compared with the peak area of the corresponding external standard to determine the EAA concentration.

Measurement of Intracellular Free Ca^{2+} Concentration ($[Ca^{2+}]_i$)

A suspension of cerebellar granule neurones (5× 10^7 cells/ml) containing 0.1% BSA was incubated at 37°C with 2 μM Fura-2 AM for 45 min. Then, cells were washed and resuspended in Ca^{2+}-free incubation solution at 10^7 cells/ml. For Ca^{2+} measurements, 0.5 ml of the cell suspension were placed in an aggregometer cuvette (Chronolog) inside the fluorimeter turret by means of an adapter. Fluorescence was measured using a Perkin-Elmer MPF44B Fluorescence spectrophotometer. Excitation and emission wavelengths were set at 340 and 490 nm respectively. $CaCl_2$ (1 mM) was added to the suspension before each experiment and cerebellar granule neurones were stirred continuously using a teflon-coated stirring bar. In some experiments EGTA (1 mM) was added instead of $CaCl_2$. For the calculation of the intracellular calcium concentration, the peak response was measured. Calibration of the fluorescent signal was performed at the end of each individual experiment: minimal fluorescence was measured after Tris/EGTA (30/4 mM) and 200 μM digitonin were added and maximal fluorescence after excess $CaCl_2$ (5 mM) was added. Intracellular concentrations were calculated as described.[29]

Measurement of Cerebellar Granule Cells Membrane Protein Thiols

The changes in membrane protein thiol content were measured colorimetrically using dithio nitrobenzoate (DTNB). Briefly, cerebellar granule cells suspended in incubation solution were incubated in the presence of 0.3 mM DTNB for 30 min at room temperature after their treatment with $ONOO^-$. Then cells were spun down at 200×g for 10 min and absorbance was measured in the supernatant at 412 nm (ε_{412nm}=13,600 $M^{-1}cm^{-1}$)[30] vs. blank consisting of DTNB in incubation solution.

Synthesis of $ONOO^-$

Peroxynitrite was synthesised by the reaction of acidified $NaNO_2$ (2.1 M) with H_2O_2 (2.1 M) in a quenched flow reactor.[31] The reaction was quenched with 4.2 M NaOH and any unreacted H_2O_2 removed with solid MnO_2. To prepare decomposed $ONOO^-$ (dec $ONOO^-$), the addition of NaOH to the $H_2O_2/NaNO_2$ mixture was delayed for 3 min, after which no $ONOO^-$ was present. Peroxynitrite was stored at −20°C before

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use. The concentration was determined prior to each experiment by measuring the absorbance at 302 nm (ε_{302nm}=1,670 $M^{-1}cm^{-1}$).[32] Typical concentrations of $ONOO^-$ were 220–350 mM. Dilutions were made into water before addition to the suspension.

Chemicals and Statistical Analyses

Fura-2 AM was from Molecular Probes, BAPTA/AM ([1,2-bis-(o-Aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl) ester] was from Alexis Corporation, dihydrokainic acid was from Tocris Cookson and other chemicals were from Sigma. Results are expressed as mean ±SEM of the indicated number of experiments, and statistical comparisons were made using a Newman-Keuls test (Pharmacologic Calculation System software, version 4.0); $p<0.05$ was considered as statistically significant.

RESULTS

Effects of $ONOO^-$ on EAA Release from Acutely Dissociated Cerebellar Granule Cells in Suspension

Peroxynitrite (100–500 μM), but not decomposed $ONOO^-$ (500 μM; Dec $ONOO^-$) caused a concentration-dependent release of aspartate (Fig. 1A) which was maximal at 400 μM. In these conditions, no glutamate release over basal was detected (Fig. 1B). The NO donor SNAP (30 μM) did not cause any net release of either aspartate (n=3–9, $p>0.05$) or glutamate (n=3–9, $p>0.05$).

Effects of EGTA, BAPTA/AM, Tetrodotoxin, Na^+ Removal, Dithiothreitol and Dihydrokainic Acid on Aspartate Release Induced by $ONOO^-$ in Cerebellar Granule Cell Suspensions

To ascertain the mechanism of $ONOO^-$-induced aspartate release, the effects of several pharmacological approaches on $ONOO^-$ (400 μM)-induced aspartate release were studied. To determine whether release was Ca^{2+}-dependent, the Ca^{2+} chelator EGTA and the intracellular Ca^{2+} chelator BAPTA/AM were used. In the presence of 1 mM EGTA, $ONOO^-$ (400 μM)-induced release was not significantly affected (Fig. 2). Similarly, when cells had been previously incubated with 10 μM BAPTA/AM, the release induced by $ONOO^-$ was not significantly affected as compared with control (Fig. 2). To check its dependence on extracellular Na^+, the Na^+ channel blocker tetrodotoxin (TTX) and the removal of Na^+ by equiosmotic substitution with choline were employed. TTX (1 μM) was found to inhibit significantly aspartate release induced by 400 μM $ONOO^-$ (Fig. 2). When Na^+ was substituted by choline, the effect of $ONOO^-$ was substantially inhibited (Fig. 2). The disulphide reducing agent dithiothreitol (DTT) and the EAA uptake inhibitor dihydrokainic acid (DHK) were applied to examine the effect of thiol oxidation state and the implication of EAA transporters, respectively. DTT (1 mM) caused an inhibition on aspartate release induced by $ONOO^-$. However, the incubation with DHK (10 mM) did not modify $ONOO^-$ (400 μM)-induced aspartate release (Fig. 2).

The ability of the cells to release EAA was intact since cells that were challenged with KCl (50 mM) released both aspartate (net release: 542 ± 48 ng/10^7 cells, n=3) and glutamate (net release: 1228 ± 84 ng/10^7 cells, n=3). The effectiveness of the Ca^{2+} chelators was shown by the fact that KCl (50 mM)-induced EAA release was significantly inhibited in the presence of 1 mM EGTA (net aspartate release: 204 ± 35 ng/10^7 cells; net glutamate release: 520 ± 61 ng/10^7 cells) and in BAPTA/AM-loaded cells (net aspartate release: 111 ± 32 ng/10^7 cells; net glutamate release: 284 ± 27 ng/10^7 cells).

Effects of $ONOO^-$ on $[Ca^{2+}]_i$ in Cerebellar Granule Cells in Suspension

To investigate further the actions of $ONOO^-$, we studied whether this molecule had any effect on

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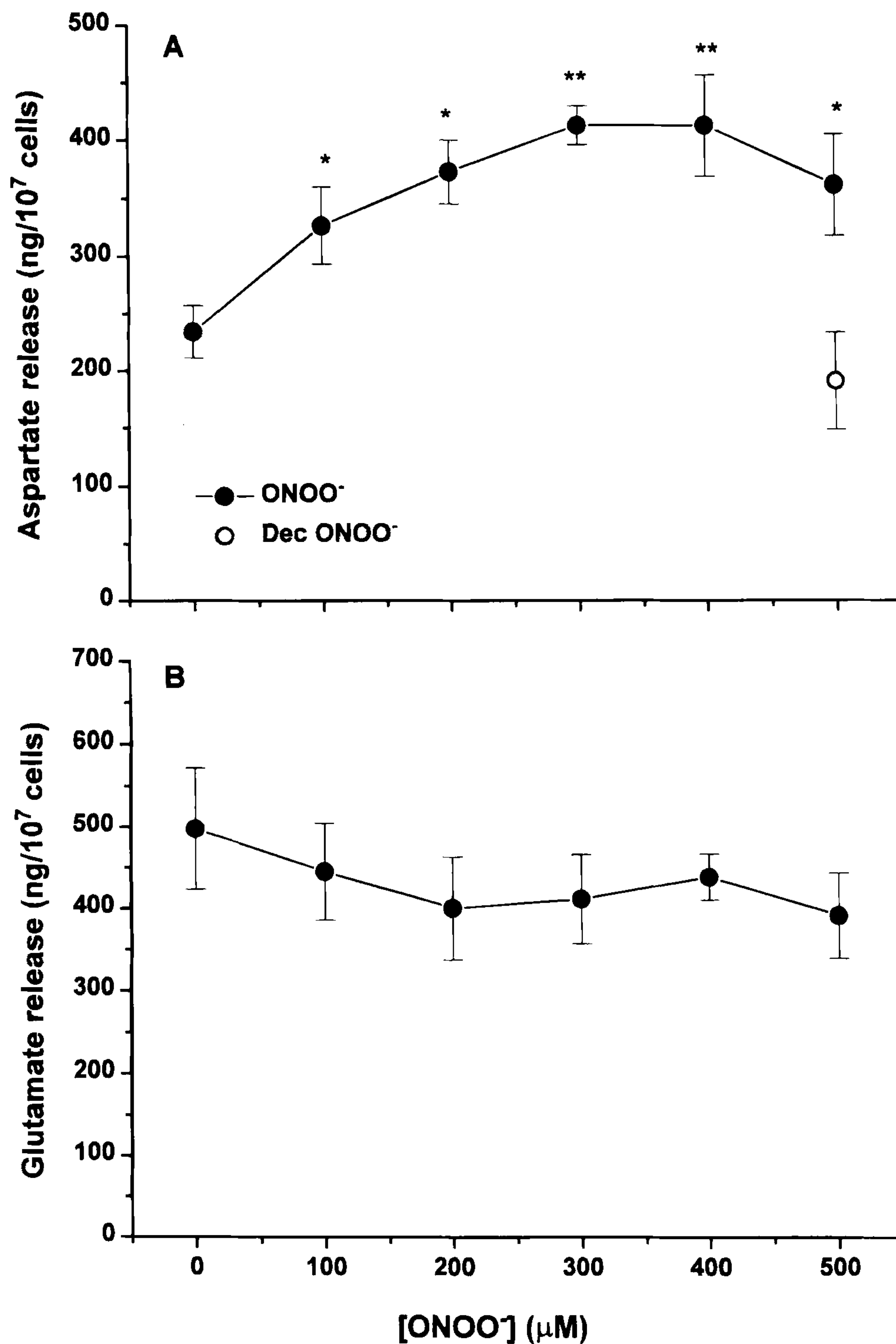

FIGURE 1 Peroxynitrite induced the release of aspartate but not of glutamate from acutely dissociated rat cerebellar granule neurones. **(A)** Peroxynitrite ($ONOO^-$; 100–500 μM) but not decomposed $ONOO^-$ (500 μM; Dec $ONOO^-$) caused the release of aspartate. **(B)** In these conditions, $ONOO^-$ did not have any significant effect on glutamate release. Cells were treated with a bolus addition of $ONOO^-$ and the supernatant was collected for excitatory amino acid determination as described under Materials and Methods. Results are means ± SEM of at least three different experiments. $^*p < 0.05$; $^{**}p < 0.01$ (Newman-Keuls test).

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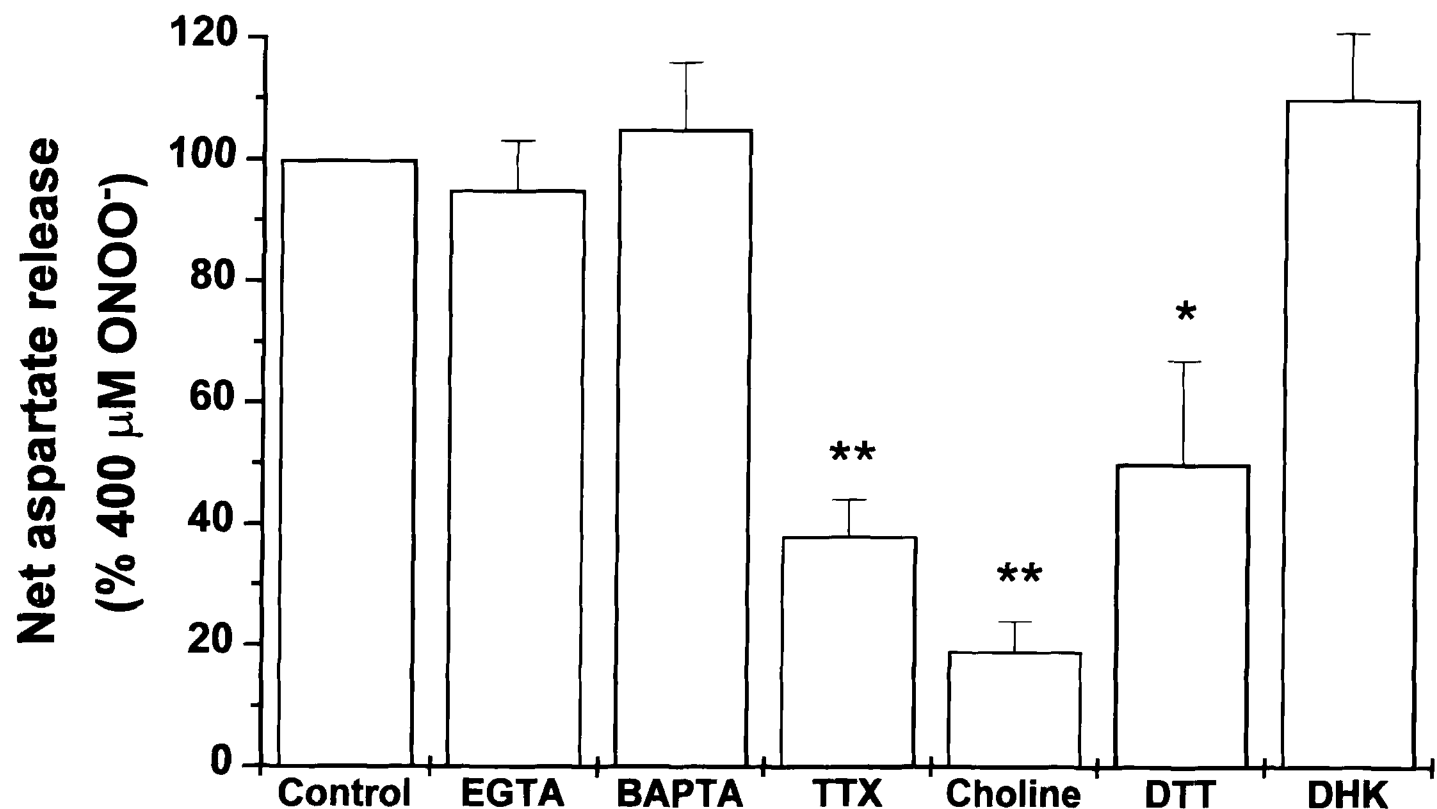

FIGURE 2 Characterisation of $ONOO^-$-induced aspartate release: Effects of EGTA (1 mM), BAPTA/AM (BAPTA, 10 μM), tetrodotoxin (TTX, 1 μM), Na^+ deprivation by equiosmotic substitution with choline (Choline), dithiothreitol (DTT, 1 mM) and dihydrokainic acid (DHK, 10 mM) on $ONOO^-$ (400 μM)-induced aspartate release (Control). Cell treatment and excitatory amino acid determination were performed as described under Materials and Methods. Results are expressed as % of $ONOO^-$ (400 μM)-induced net aspartate release and are means ± SEM of at least three different experiments. $^*p<0.05$; $^{**}p<0.01$ (Newman-Keuls test).

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$[Ca^{2+}]_i$ in cerebellar granule cell suspensions. The basal $[Ca^{2+}]_i$ in this preparation was found to be 87 ± 5 nM ($n=20$). Peroxynitrite (100–500 μM) caused a concentration-dependent increase in $[Ca^{2+}]_i$ over the basal levels (Fig. 3), reaching a maximum of 367 ± 69 nM at 500 μM $ONOO^-$ when the peak concentration was measured. Decomposed $ONOO^-$ (500 μM) did not have any significant effect. When the experiment was performed in the presence of 1 mM EGTA, $ONOO^-$ (300 μM) elicited an increase in $[Ca^{2+}]_i$ which was not significantly inhibited when compared with that obtained in the absence of EGTA ($\Delta[Ca^{2+}]_i=$ 110 ± 24 nM vs 62 ± 19 nM, in the absence and presence of EGTA, respectively; $n=4$–8, $p>0.05$).

Effects of $ONOO^-$ on Cerebellar Granule Cell Membrane Protein Thiols

Since DTT was able to inhibit $ONOO^-$ (400 μM)-induced aspartate release, we decided to study the effects of $ONOO^-$ on protein membrane thiols in intact cells after the addition of this oxidant. Protein membrane thiol concentration was found to be 44.2 ± 0.8 μM ($n=4$) for a suspension containing 10^7 cells/ml. Consistently with the action of DTT, $ONOO^-$ (100–500 μM), but not decomposed $ONOO^-$ (500 μM, Fig. 4) caused a concentration-dependent decrease in the concentration of protein membrane thiols (Fig. 4).

DISCUSSION

We have studied the effects of $ONOO^-$ on cerebellar granule neurones in suspension, with continuous stirring, in order to allow a direct action of $ONOO^-$ on its cellular targets and diminish the interactions with the bathing solutions. We have found several effects of $ONOO^-$ on this preparation: 1) this molecule causes a concentration-dependent release of aspartate which was partially or totally inhibited by DTT, TTX, and in Na^+-deprived solutions and not affected by EGTA, BAPTA or dihydrokainic acid; 2) $ONOO^-$ evokes a concentration-dependent increase in $[Ca^{2+}]_i$ and 3) a concentration-dependent depletion of membrane protein -SH groups.

Regarding the mechanism of release of aspartate, we have found that it is not affected by the chelation of either extracellular or cytosolic Ca^{2+}, which strongly suggests that $ONOO^-$-induced aspartate release is not exocytotic. These neurones are in early stages of development, but we have shown that they are able to release both aspartate and glutamate when challenged with stimuli that raise intracellular Ca^{2+} and lead to exocytosis, such as depolarisation by high K^+. Interestingly, Ca^{2+} removal did not completely inhibit K^+-induced EAA release, in agreement with previous data in the literature showing that K^+-induced EAA release is partially independent of Ca^{2+} in 1-week-old rat cerebellar granule cell cultures.[33]

A possible explanation for $ONOO^-$-induced aspartate release is that it is due to a nonvesicular efflux by reversal of a Na^+-dependent plasma membrane transporter of this EAA.[34–36] This mechanism of release may result from a large decrease in the electrochemical Na^+ gradient caused by $ONOO^-$. Indeed, $ONOO^-$ has been reported to change the membrane permeability to Na^+ ions.[37] This is consistent with our findings showing that $ONOO^-$-induced aspartate release is blocked in solutions in which Na^+ has been substituted by choline. The inhibitory effect caused by TTX suggests that Na^+ channels might be implicated. However, both the lack of effect of dihydrokainic acid and the absence of glutamate release in these conditions are difficult to explain: since dihydrokainic acid blocks EAA transporters, release due to transporter reversal should have been inhibited in the presence of this molecule. An interpretation might be that a selective and dihydrokainic acid-insensitive L-aspartate transporter is involved and that L-glutamate efflux is not observed due to the fact that its transporter is inhibited by $ONOO^-$, as it has been shown previously.[38] On the other hand, exocytotic release of glutamate would be expected

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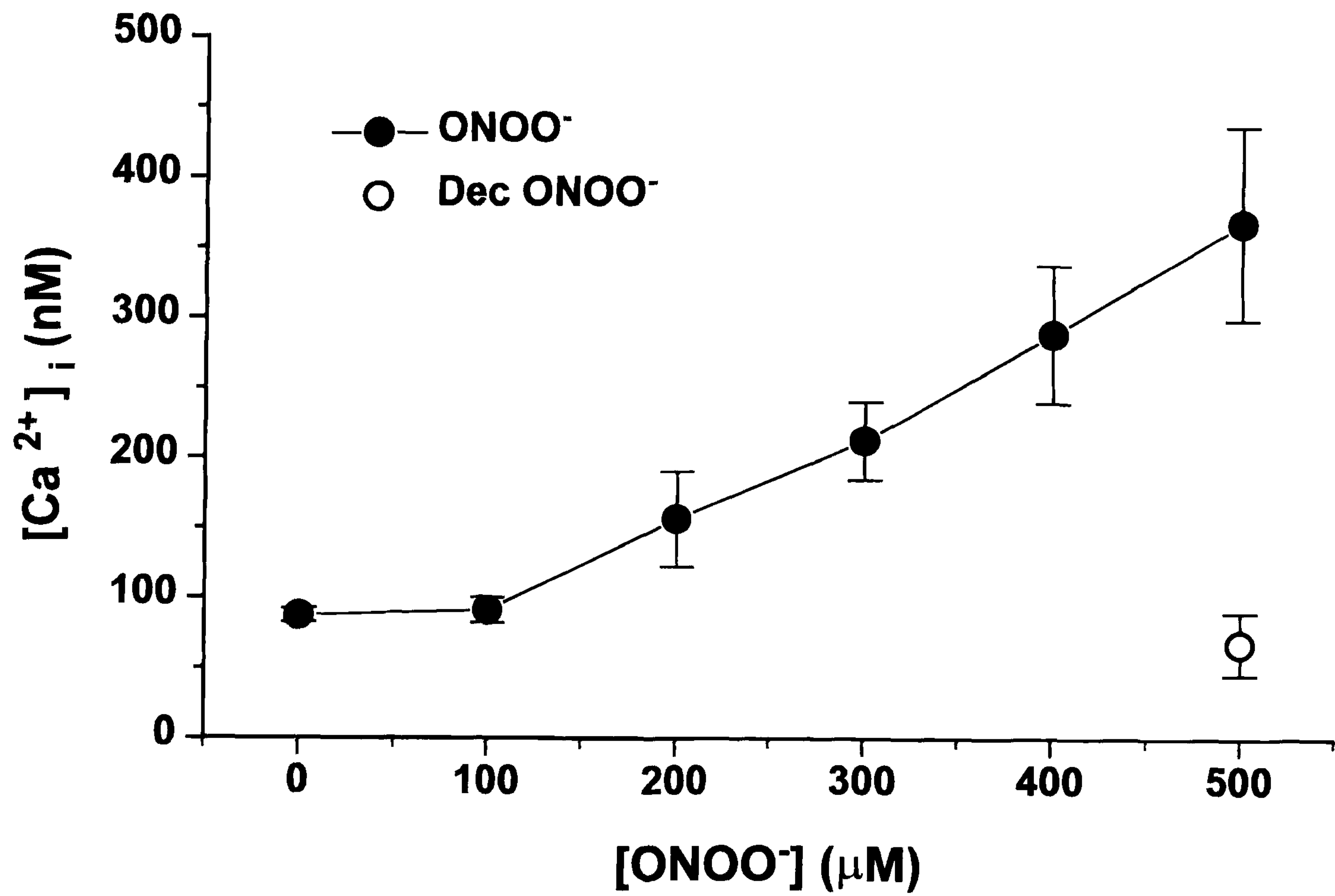

FIGURE 3 Effect of $ONOO^-$ on $[Ca^{2+}]_i$ in Fura-2-loaded cerebellar granule neurones in suspension. Cell treatment and $[Ca^{2+}]_i$ determination were performed as described under Materials and Methods. Results correspond to the peak response and are means $\pm$ SEM of at least three different experiments.

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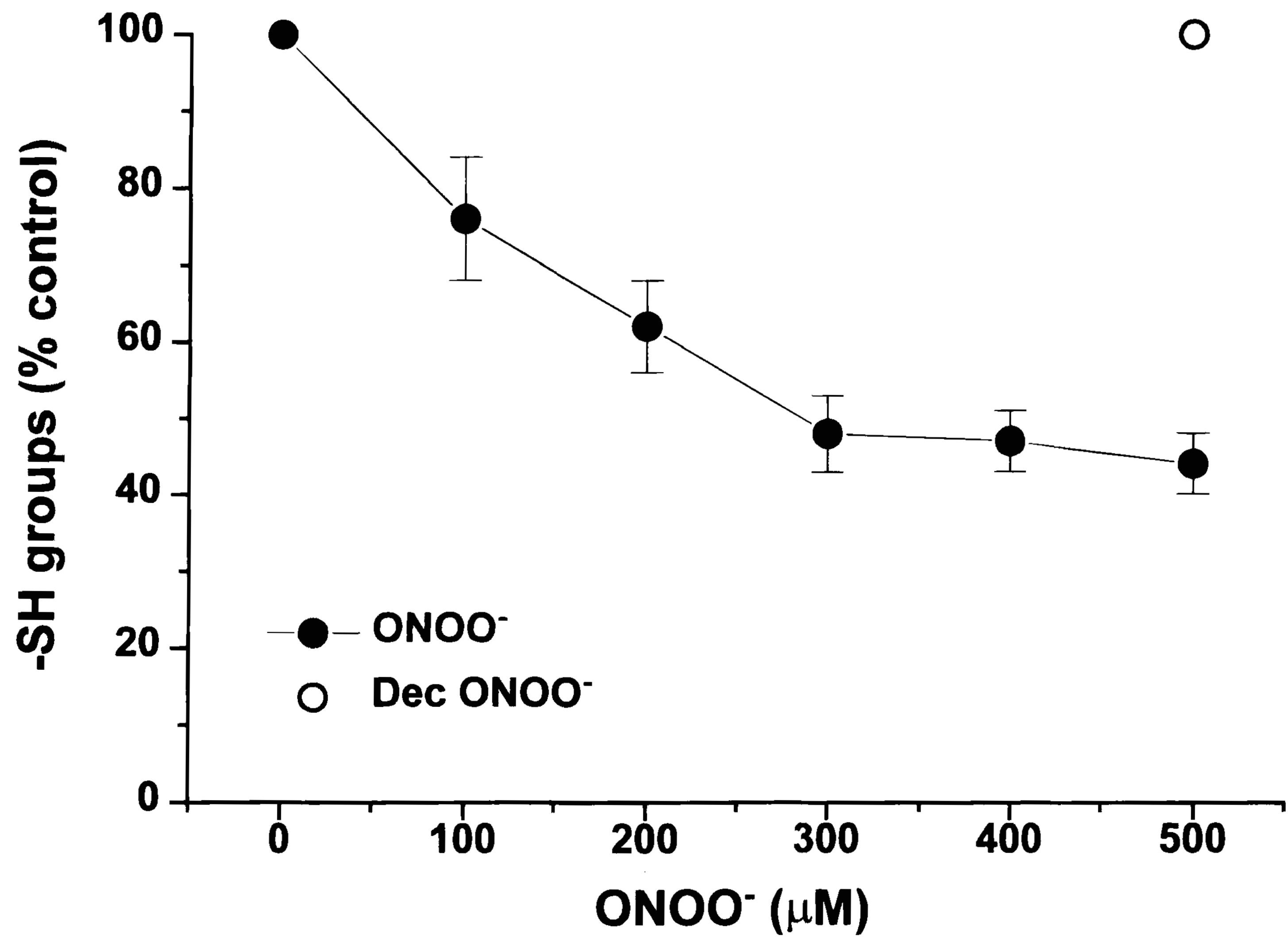

FIGURE 4 Effects of $ONOO^-$ in protein membrane thiol concentration in rat cerebellar granule neurones. Thiol group concentration on intact cells treated with $ONOO^-$ was measured spectrophotometrically as described under Materials and Methods. Results are expressed as % of total thiol content in untreated cells and are means ± SEM of at least three different experiments.

from the elevation in intracellular Ca^{2+} observed in Fura-2-loaded cells. However, the Ca^{2+} concentration able to drive synaptic vesicle fusion corresponds to the very high level achieved only within the microdomain of elevated Ca^{2+} near the inner mouth of open calcium channels.[39] Our method does not discriminate the features of $ONOO^-$-induced $[Ca^{2+}]_i$ elevation and it might occur that it is inefficient to provide the local levels of this cation necessary to evoke exocytosis.

Aspartate release induced by peroxynitrite is partially inhibited by DTT, suggesting an involvement of thiol oxidation in this process. The simultaneous decrease in membrane thiols found in these conditions is consistent with this idea. The partial effect of DTT to inhibit $ONOO^-$-induced aspartate release might be due to the fact that some of the products of $ONOO^-$-induced thiol oxidation are beyond sulfenic acid, therefore not reducible by DTT[5], although thiol-independent mechanisms, such as nitrosylation and/or nitration of aromatic amino acids, might also be involved.

On the other hand, we have shown that $ONOO^-$ increases $[Ca^{2+}]_i$ in this preparation. This elevation was not significantly affected by EGTA, suggesting that $ONOO^-$ induces a release of Ca^{2+} from intracellular stores. Although calcium mobilisation might be expected from an action of $ONOO^-$ on mitochondrial respiration due to irreversible inhibition of complexes I–III and II–III caused by $ONOO^-$,[40,41] we have previously demonstrated that $ONOO^-$ must be formed in the close vicinity of the mitochondria to be able to cause this effect.[41] An alternative explanation is that the persistent increase in the intracellular Na^+ concentration impairs the ability of neurones to extrude Ca^{2+} via the Na^+/Ca^{2+} exchanger contributing to the destabilisation of Ca^{2+} homeostasis.[42] Recently it has been shown that NO disrupts Ca^{2+} homeostasis in hippocampal neurones.[43] This mechanism might play a role in our preparation, since we have demonstrated that $ONOO^-$ is very efficiently detoxified by cellular antioxidant defences leading to molecules with the ability to donate NO.[11,44] However, we think that this mechanism is not involved in the phenomenon we report here, since the NO donor SNAP did not increase either aspartate release or intracellular Ca^{2+} concentration (data not shown) in our preparation.

Excitatory amino acids interact with several types of receptors in the vertebrate CNS; among them, the *N*-methyl-D-aspartate (NMDA) receptor is the best defined subtype and its activation is essential to most EAA neurotoxicity. Interestingly, the NMDA receptor can be selectively activated by L-aspartate[18] and, although glutamate is the main excitatory amino acid, a selective release of aspartate has been reported in some circumstances.[45,46] In addition to its oxidising properties, our data suggest that $ONOO^-$, by inducing the efflux of L-aspartate from neurones, may exacerbate NMDA-mediated excitotoxic damage in conditions such as brain ischaemia. Moreover, the excitotoxic damage may be further aggravated by the increase in intracellular Ca^{2+} concentration induced by $ONOO^-$ in these cells.

In summary, we have described that extracellularly formed $ONOO^-$ causes aspartate release by a Ca^{2+}-independent mechanism and also a concomitant increase in $[Ca^{2+}]_i$ in acutely dissociated cerebellar granule neurones. These findings may provide new leads that permit better understanding of the mechanisms of NO-induced neuronal damage in pathological circumstances.

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