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The Cu–Zn superoxide dismutase (SOD1) inhibits ERK phosphorylation by muscarinic receptor modulation in rat pituitary GH3 cells

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ABSTRACT

The Cu–Zn superoxide dismutase (SOD1) belongs to a family of isoenzymes that are able to dismutate the oxygen superoxide in hydrogen peroxide and molecular oxygen. This enzyme is secreted by many cellular lines and it is also released trough a calcium-dependent depolarization mechanism involving SNARE protein SNAP 25. Using rat pituitary GH3 cells that express muscarinic receptors we found that SOD1 inhibits P-ERK1/2 pathway trough an interaction with muscarinic M1 receptor. This effect is strengthened by oxotremorine, a muscarinic M agonist and partially reverted by pyrenzepine, an antagonist of M1 receptor; moreover this effect is independent from increased intracellular calcium concentration induced by SOD1. Finally, P-ERK1/2 inhibition was accompanied by the reduction of GH3 cell proliferation.

These data indicate that SOD1 beside the well studied antioxidant properties can be considered as a neuromodulator able to affect mitogen-activated protein kinase in rat pituitary cells trough a M1 muscarinic receptor.

All eukaryotic cell surface receptors respond to extracellular signals through transducing mechanisms involving extracellular signal regulated protein kinase or mitogen-activated protein kinases (MAPKs) pathway [1].

Rat pituitary GH3 cells express voltage-dependent calcium channels (VDCC) and show Ca^{2+} oscillations, therefore they represent a well-known line of excitable cells [2] strictly correlated to VDCC. Previous studies demonstrated that SOD1 is secreted by many cellular lines [3] enclosed neuroblastoma SK-N-BE cells trough an ATP-dependent mechanism [4]; the SOD1 export is able to interact with cell membrane of neuroblastoma cells activating a phospholipase C/protein kinase C pathway; this effect is coupled to an increase of intracellular Ca^{2+} concentration [5]. Very recently we demonstrated that the depolarization of GH3 cells and rat brain synaptosomes, induced by 55 mM/L K^+, determined a release of SOD1 that was abolished by the calcium chelator EDTA or by botulinum toxin A that cleaves SNAP-25 protein [6].

Together these data indicate that, in addition to the constitutive SOD1 secretion, this enzyme is also released by depolarization trough calcium-dependent mechanisms.

It is well known that muscarinic acetylcholine receptors mediate diverse physiological functions. At present, muscarinic receptors can be divided in five subtypes (M(1)–M(5); M(1), M(3), and M(5) receptors are preferentially coupled to G(q/11) and activate phospholipase C, which initiates the phosphatidylinositol trisphosphate cascade leading to intracellular Ca^{2+} mobilization and activation of protein kinase C [7–9].

M receptors are expressed in pituitary GH3 cells [10] and are remarkably involved in the modulation of ERK activation through phospholipase C–protein kinase C pathway; therefore in the present study we aim to investigate whether SOD1 administration in rat pituitary GH3 cells is able to affect ERK1/2 transductional pathway through M receptors.

Materials and methods

Cell cultures. Rat pituitary GH3 cells, obtained from Flow Laboratories (Irvine, UK), were grown in a humidified 5% CO_2 atmosphere in dishes containing F10 medium (Gibco, Life Technology, Carsband, CA, USA) supplemented with 10% foetal bovine serum, 15% HS (Gibco, Life Technology), 100 IU of penicillin/mL and 100 μg of streptomycin/mL (Sigma, St. Louis, MO, USA).

Immunoblotting assay. Western blotting was performed as previously described [4]. Briefly GH3 cells, washed twice with cold phosphate-buffered saline and scraped from the plate, were homogenized in buffer containing 10 mM Tris–HCl, pH 7.4, 4 mM

Abbreviations: SOD1, Cu–Zn superoxide dismutase; MAPK, mitogen-activated protein kinase; GH3, rat pituitary cells; $[Ca^{2+}]_i$, intracellular calcium concentration; EDTA, ethylenediaminetetraacetic acid; Fura-2AM, Fura-2 acetoxymethyl ester; SDS, sodium dodecyl sulphate.

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2-mercaptoethanol, 5 mM EDTA and a mix of protease inhibitors. Nuclei and cell debris were eliminated by slight centrifugation at 3000 rpm for 5 min; the supernatant was centrifuged at 100,000×g for 1 h at 4 °C.

After centrifugation the supernatant (cytosol) was collected and 50 μg of it was electrophoresed on 12% sodium dodecyl sulphate (SDS)–polyacrylamide gel. Standard procedures were used for western blotting experiments using enhanced chemiluminescent detection according to the manufacturer's instructions (Amersham Life Science, UK).

Primary rabbit anti-human Cu–Zn SOD antibodies (Santa Cruz Biotechnology, CA, USA) were used at 1:1000 dilution, while the secondary antibody, a horseradish peroxidase-linked donkey anti-rabbit IgG, was used at 1:2000 dilution.

$[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ was measured by single-cell computer-assisted videoimaging [11]. Briefly, GH_3 cells, grown on glass coverslips, were loaded with 6 μM Fura-2 acetoxymethyl ester (Fura-2AM, EMD Biosciences) for 30 min at 37 °C in normal Krebs solution containing the following (in mM): 5.5 KCl, 160 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes–NaOH, pH 7.4. At the end of the Fura-2AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto a Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40× oil objective lens. The experiments were carried out with a digital imaging system composed of MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton, NJ, USA), LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, CA, USA), and Meta-Morph/MetaFluor Imaging System software (Universal Imaging, West Chester, PA, USA). After loading, cells were alternatively illuminated at wavelengths of 340 nm and 380 nm by a Xenon lamp. The emitted light was passed through a 512-nm barrier filter. Fura-2 fluorescence intensity was measured every 3 s. Forty to sixty-five individual cells were selected and monitored simultaneously from each cover slip. Results are presented as the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Calibrations utilized the relation of [12] assuming that the K_D for FURA-2 was 224 nM.

Cell viability. Cell viability, determined by Lactate dehydrogenase (LDH) activity in the incubation medium of GH3 cells, was performed by Roche Molecular Biochemical kit (Mannheim, Germany).

Cell proliferation evaluation by [^{3}H]thymidine incorporation. GH_3 cells were grown for 24 h in their culture medium containing foetal bovine serum and HS. After 30′ exposure to 150 ng/ml of SOD1 (Sigma, Aldrich, Italy) or normal Krebs solution, cells were growth for 24, 60 and 72 h in the presence of 2 μCi/well of [3H]thymidine (Amersham). Then, cells were harvested for further 6 h before counting them in a β counter (Beckman LS 6500). Each experiment was repeated three times.

Results

Characterization of M-receptor activity in GH_3 cells and Effect of SOD1 on $[Ca^{2+}]_i$ homeostasis

The role of muscarinic receptors in the control of calcium homeostasis has been characterized by their pharmacological modulators in pituitary GH_3 cells. The agonist oxotremorine (10 μM) was able to induce $[Ca^{2+}]_i$ increase that was partially prevented by the M1-receptor antagonist pyrenzepine (15 μM) (Fig. 1A and B). These results suggested the presence of other subtypes of muscarinic receptors in pituitary GH3 cells.

The role of SOD1 in calcium homeostasis has also been investigated. Particularly, SOD1 (150 ng/ml) induced a significant increase in $[Ca^{2+}]_i$ that was partially prevented by the specific

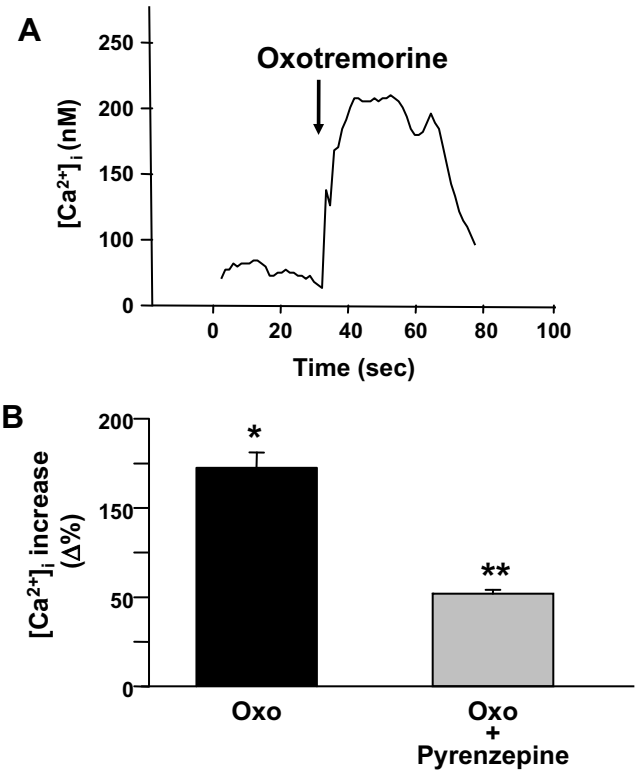

Fig. 1. Effect of the muscarinic agonist oxotremorine on $[Ca^{2+}]_i$ in pituitary GH_3 cells. (A) single-cell trace representative for the effect of oxotremorine (10 μM) on $[Ca^{2+}]_i$. (B) Quantification of the effect exerted by oxotremorine (10 μM) and oxotremorine (10 μM) plus pyrenzepine (15 μM) on $[Ca^{2+}]_i$. $^{*}p < 0.05$ vs Ctl values. $^{**}p < 0.05$ vs oxotremorine. Each bar represents the mean ± SEM of almost 60 cells recorded in three independent experimental sessions.

SERCA inhibitor thapsigargin or by Ca^{2+}-free solution (Fig. 2C). These data suggested that the SOD1-mediated modulation of $[Ca^{2+}]_i$ homeostasis was exerted both at intracellular and extracellular levels.

Effect of SOD1 on MAPK1/2 pathway in GH_3 cells

To investigate the role of extracellular SOD1 (150 ng/ml) on MAPK pathway activation in GH_3 cells, we analysed the effect of SOD1 on ERK1/2 phosphorylation at different times of incubation (Fig. 3A and B). The time course of SOD1 administration showed a marked inhibition of P-ERK that picked at 30 min of incubation (Fig. 3). The M-receptor agonist oxotremorine (10 μM) reproduced the same effect of SOD1 (150 ng/ml) on MAPK pathway by inhibiting ERK phosphorylation in a time-dependent way (Fig. 3C and D); as can be noticed, the co-incubation for 30 min of SOD1 with oxotremorine strongly potentiated the inhibitory effect on P-ERK. To evaluate the role of M1 receptor on the SOD1-induced modulation of ERK1/2 phosphorylation, we used the well-known M1-antagonist pyrenzepine (15 μM) together with SOD1 (150 ng/ml). This M1-antagonist was able to partially prevent the inhibitory action exerted by the superoxide dismutase 1 on P-ERK1/2 in GH3 cells (Fig. 4), thus showing the involvement of this receptor subtype in SOD1-mediated effect.

Effect of SOD1 incubation on cell proliferation

ERK phosphorylation is involved in the modulation of cell proliferation in several cellular systems and the inhibition of this path-

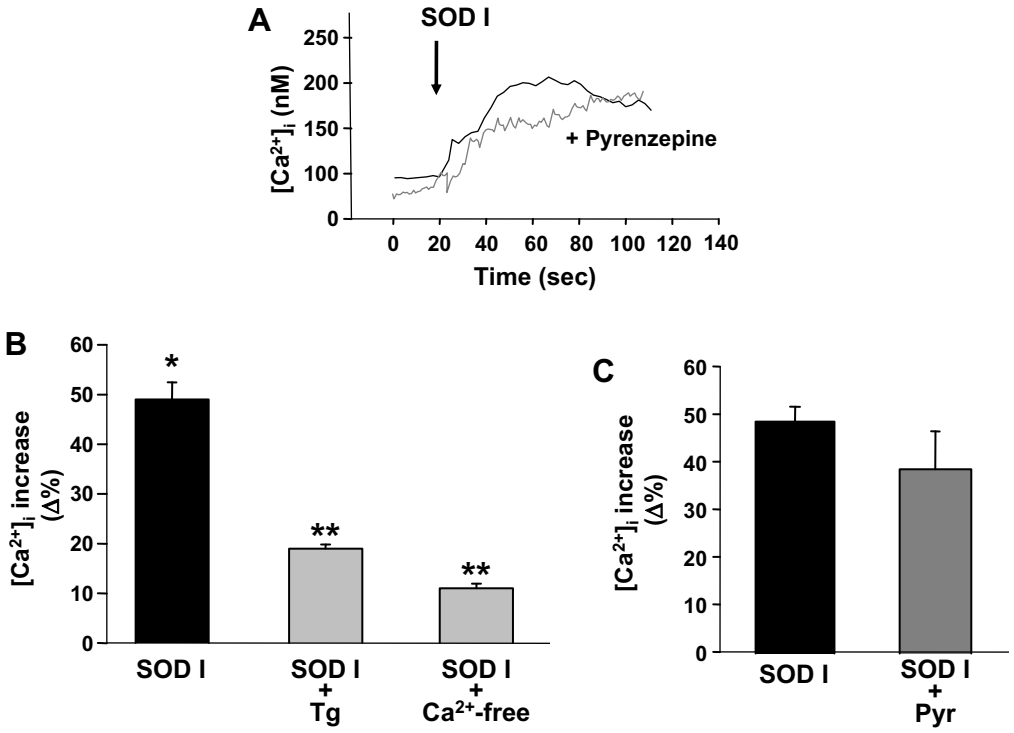

Fig. 2. Effect of SOD1 on $[Ca^{2+}]_i$ in pituitary GH_3 cells. (A) Superimposed single-cell traces representative for the effect of SOD1 (150 ng/ml) and SOD1 (150 ng/ml) plus pyrenzepine (15 μM) on $[Ca^{2+}]_i$. (B) Quantification of the effect exerted by SOD1 (150 ng/ml) and SOD1 (150 ng/ml) plus the SERCA inhibitor thapsigargin (1 μM) or Ca^{2+}-free solution on $[Ca^{2+}]_i$. $^{*}p < 0.05$ vs Ctl values; $^{**}p < 0.05$ vs SOD1 (C) Quantification of the effect exerted by SOD1 (150 ng/ml) and SOD1 (150 ng/ml) plus pyrenzepine (15 μM) on $[Ca^{2+}]_i$. Each bar represents the mean ± SEM of almost 60 cells recorded in three independent experimental sessions.

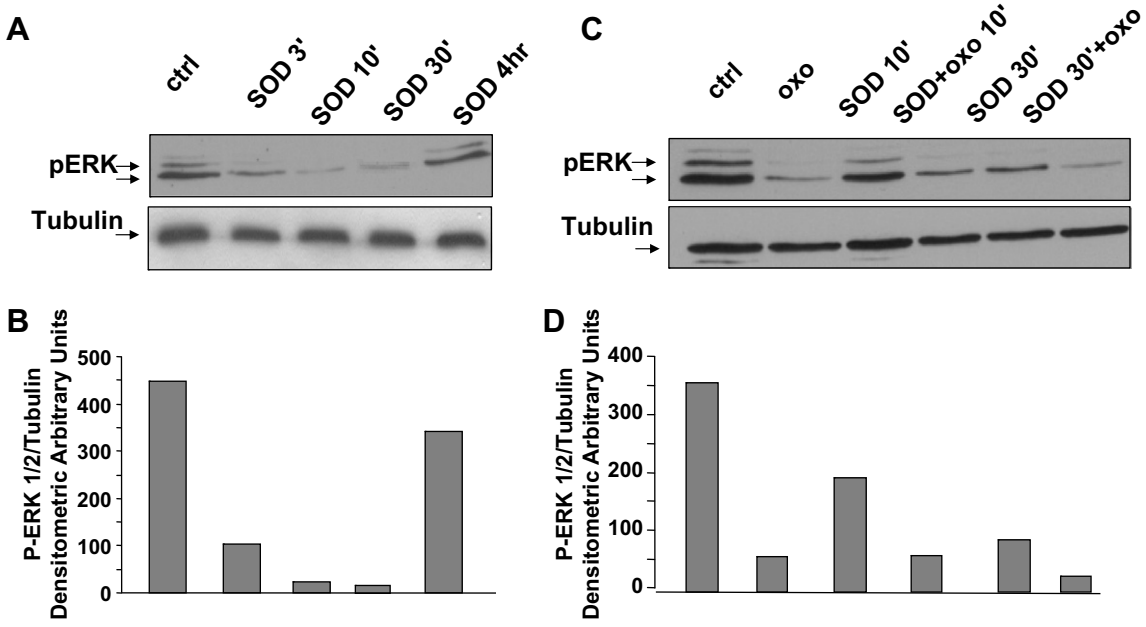

Fig. 3. Effect of SOD1 and oxotremorine on P-ERK in pituitary GH_3 cells. (A,B) Western blots of P-ERK1/2 and tubulin after different time exposure to SOD1 (150 ng/ml) and the relative densitometric analysis, respectively. (C,D) Western blots of P-ERK1/2 and tubulin after different time exposure to oxotremorine (10 μM) and oxotremorine plus SOD1 (150 ng/ml) and the relative densitometric analysis, respectively. All the data are reported as arbitrary units (normalized on the basis of the respective tubulin levels) of a single experiment repeated at least three times.

way, trough the stimulation of the cyclin-dependent kinase (Cdk) inhibitor p27, causes the reduction of pituitary cell growth [13]. In order to evaluate the role of SOD1 in GH3 cell proliferation we measured [^{3}H]thymidine incorporation at different times (24, 60, and 72 h). As can be noticed, the treatment with SOD1 (150 ng/ml) for only 30′ was able to induce a significant reduction in cell proliferation after 72 h of observation, an effect possibly linked to P-ERK inhibition (Fig. 4C).

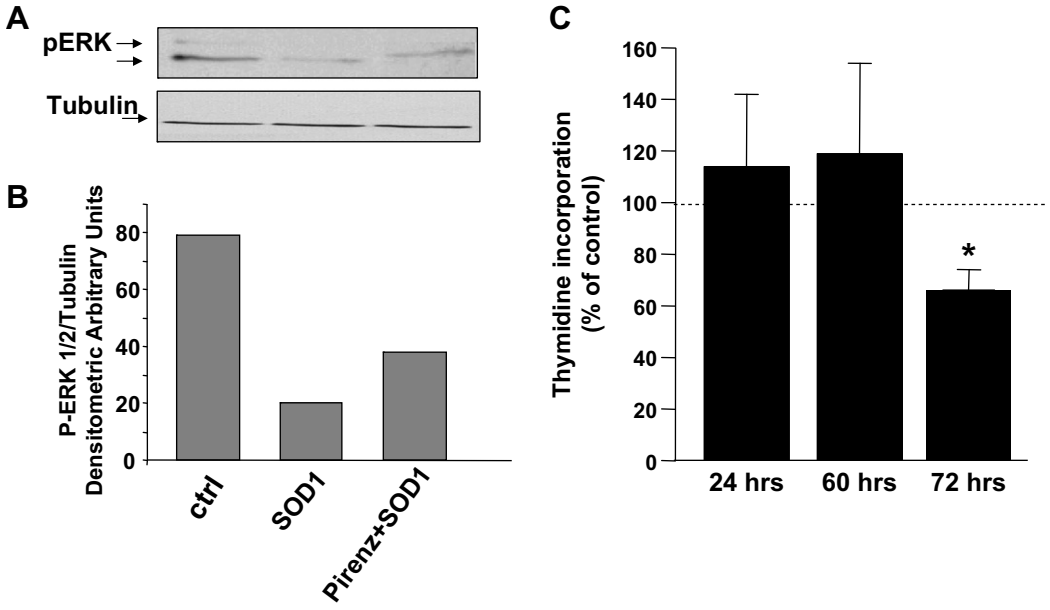

Fig. 4. Effect of the M1-antagonist pyrenzepine on SOD1-induced down-regulation of P-ERK in pituitary GH_3 cells. (A, B) Western blots of P-ERK1/2 and tubulin after the exposure for 30′ to SOD1 (150 ng/ml) and SOD1 plus pyrenzepine (15 μM) (preincubated for 10′) and the relative densitometric analysis, respectively. (C) Quantification of [^{3}H]thymidine incorporation in GH3 cells at different time after 30′ exposure to SOD1 (150 ng/ml). All the data are expressed as means ± SEM ($n = 3$). $^*p < 0.05$ vs respective Ctl.

Discussion

The secretion of SOD1 by many cellular lines has been previously reported [3,4,14] and then confirmed by Turner and Atkin [15] in NSC-34 mice motor neuron; moreover they demonstrated that a chronic intraspinal infusion of wild-type SOD1 delayed the progression of disease in transgenic $SOD1^{G93A}$ mice suggesting that an impaired SOD1 secretion is present in familiar amyotrophic lateral sclerosis linked to SOD1 mutants.

It has been also shown that the incubation of neuroblastoma SK-N-BE cells with human SOD1 is able to activate phospholipase–protein kinase C pathway increasing intracellular calcium [5]. Very recently [6], we demonstrate that, in addition to the constitutive SOD1 secretion, depolarization of pituitary rat GH3 cells and rat brain synaptosomes, by high potassium concentration, induces an additional rapid calcium-dependent secretion operated by SNARE complexes.

These double pathways of SOD1 secretion [16] opens many intriguing questions about the physiological role that could be ascribed to SOD1 export. In this study we pointed out that SOD1, besides the well-known role of antioxidant enzyme able to dismutase oxygen radical to hydrogen peroxide and molecular oxygen, carries out a new function trough an activation of muscarinic M1 receptor that produces an inhibition of P-ERK1/2 pathway. In particular, this effect, that was associated with an inhibition of cell proliferation in pituitary GH3 cells, cannot be ascribed to the increase in intracellular calcium concentration induced by SOD1. In fact, the incubation of GH_3 cells with the M1 antagonist pyrenzepine failed to prevent $[Ca^{2+}]_i$ increase elicited by SOD1 whereas this molecule was able to prevent phosphorylation of-ERK1/2 in the same experimental conditions. Therefore, this evidence suggests the existence of two different pathways elicited by SOD1 in pituitary GH3 cells.

As concerns the effect exerted by SOD1 on cell proliferation, it was similar to those exerted by somatostatin and its analogues in pituitary tumours [17]. This evidence further suggests a paracrine neuromodulatory role exerted by this antioxidant enzyme.

In conclusion, this study demonstrated that SOD1, through an interaction with muscarinic receptors, carried out a down-regulation of P-ERK1/2 pathway associated with a reduction of GH3 cell proliferation.

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