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Vanadate Protects Human Neuroblastoma SH-SY5Y Cells Against Peroxynitrite-Induced Cell Death

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Abstract We investigated the effect of vanadate, a tyrosine phosphatase inhibitor, on cell death induced by peroxynitrite in human neuroblastoma SH-SY5Y cells. Vanadate prevented cell death induced by 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor; whereas SIN-1-induced cell death was not prevented by neither okadaic acid, an inhibitor of serine/threonine phosphatases 1 and 2A, nor cyclosporin A, an inhibitor of serine/threonine phosphatase 2B. Vanadate did not prevent cell death induced by N-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethanamine, a nitric oxide donor. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), did not block the protective effect of vanadate, suggesting that the protective effect of vanadate is independent on PI3-kinase. Vanadate increased tyrosine phosphorylation of several proteins including the focal adhesion protein p130 Crk-associated substrate ($p130^{cas}$). By the treatment with SIN-1, the endogenous association of $p130^{cas}$ and Crk was disrupted, and the association was restored by vanadate treatment. These results suggest that disruption of tyrosine phosphorylation signaling may be critical for peroxynitrite-induced cell death, and that vanadate prevents cell death at least in part through the enhancement in tyrosine phosphorylation of the proteins including $p130^{cas}$. J. Cell. Biochem. 85: 721–727, 2002.

Key words: vanadate; tyrosine phophorylation; nitrotyrosine; peroxynitrite; cell death; SH-SY5Y

Peroxynitrite is a strong oxidant produced by a reaction of superoxide with nitric oxide [Beckman et al., 1990]. There is increasing evidence to suggest that peroxynitrite is involved in pathogenesis of neurodegenerative disorders, such as Parkinson's disease [Good et al., 1998]. Although some mechanisms of peroxynitrite-induced cell death have been proposed, crucial pathways involved in peroxynitrite-induced cell death are still unclear.

Vanadate is a widely-used tyrosine phosphatase inhibitor and causes a wide variety of biological responses. It has insulin-mimetic effects and has been shown to be potentially useful in treating both insulin-dependent and non-insulin-dependent diabetes mellitus [Brichard and Henquin, 1995]. Although the mechanism by which vanadate mimics insulin is not completely clear, recent evidence suggests that tyrosine phosphatase inhibition and subsequent activation of tyrosine phosphorylation signaling are important [Shechter et al., 1995]. One of such signaling molecules is phosphatidyl-inositol 3-kinase (PI3-kinase). For example, vanadate activates PI3-kinase in rat adipocytes and hepatocytes [Band and Posner, 1997].

Signaling pathways involved in neuronal survival have been extensively characterized. The role for PI3-kinase has been established, especially in growth factor-induced promotion of cell survival. For example, PI3-kinase has been shown to be implicated in the signaling of insulin-like growth factor-1 (IGF-1)-mediated protection of cell death [Yao and Cooper, 1995]. Our previous studies also indicate that IGF-1 protects against peroxynitrite-induced cell death via PI3-kinase in human neuroblastoma SH-SY5Y cells [Saeki et al., 2002].

Abbreviations used: Crk, CT-10 regulated kinase; ECM, extracellular matrix; FAK, focal adhesion kinase; IGF-1, insulin-like growth factor-1; SH2, Src homology 2; $p130^{cas}$, p130 Crk-associated substrate; PI3-kinase, phosphatidylinositol 3-kinase.

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Another survival pathway mediated by tyrosine phosphorylation is cell adhesion signaling. It has been reported that loss of attachment to extracellular matrix (ECM) causes cell death in many cell types [Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997]. Although the signaling cascade regulating survival signals from ECM has not been completely elucidated, several important effector molecules have been identified. Upon stimulation, tyrosine kinases such as focal adhesion kinase (FAK) and Src are activated, then FAK/Src phosphorylates p130 Crk-associated substrate ($p130^{cas}$), which serves as a scaffold for the recruitment of various adaptor proteins, such as Crk [Schlaepfer and Hunter, 1998].

Since tyrosine phosphorylation plays an important role in neuronal cell death, we examined the effect of vanadate on peroxynitrite-induced cell death in SH-SY5Y cells. We report here that vanadate may possess protective effects against peroxynitrite-induced cell death through PI3-kinase-independent mechanisms.

MATERIALS AND METHODS

Materials

3-Morpholinosyndnonimine (SIN-1) and N-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethanamine (NOC12) were purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (Logan, UT). RPMI1640 medium, cyclosporin A, okadaic acid, wortmannin, LY294002, and vanadate were from Sigma (St. Louis, MO). Amphotericin B was from GIBCO BRL (Gaithersburg, MD). Anti-$p130^{cas}$ antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Crk antibody was from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine antibodywas from Chemicon International, Inc. (Temecula, CA). Other chemicals used were commercially available and of analytical grade.

Cell Culture and Treatment

SH-SY5Y cells were cultured in RPMI1640 supplemented with 10% FBS containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 1 μl/ml amphotericin B (complete RPMI). All experiments were performed with complete RPMI. For analysis of neurotoxicity, cells were plated on 35-mm culture dish at 5×10^4 cells/cm^2. For immunoblotting, cells were plated on 10-cm culture dish at 5×10^4 cells/cm^2. Twenty-four hours after plating, vanadate was added to the medium 30 min before the addition of SIN-1. After 24-h treatment with SIN-1, cell death was evaluated using a trypan blue exclusion test or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The percentage of cell death was expressed as the percentage of stained cells as a fraction of the total number of cells. Approximately 1,000 cells were counted for each data. MTT assay was performed as described previously [Kanesaki et al., 1999].

Immunoprecipitation and Immunoblotting

For immunoprecipitation and immunoblotting, following treatment with various drugs, cells were washed twice with ice-cold phosphate-buffered saline (PBS), before the addition of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM vanadate and incubated on ice for 30 min. After centrifugation at 10,000*g* for 20 min at 4°C, equal protein concentrations of lysates were immunoprecipitated or analyzed directly by SDS–PAGE. After incubation on ice with 3 μg of antibodies for 16 h, immunoprecipitates were mixed with Protein G Sepharose (Pharmacia) for 1–2 h. The sepharose beads were washed five times with lysis buffer, associated proteins were recovered by boiling for 5 min in Laemmli buffer [Laemmli, 1970], separated by 7.5% SDS–PAGE and subjected to immunoblotting. Primary antibodies were detected using biotinylated anti-mouse IgG, followed by enhanced chemiluminescence (Wako, Osaka, Japan).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical differences between groups were determined using Student's *t*- or Tukey test after analysis of variance. $P < 0.05$ was considered significant.

RESULTS

Vanadate Protects Peroxynitrite-Induced Cell Death

We examined the effect of vanadate, tyrosine phosphatase inhibitor, on peroxynitrite-induced cell death in SH-SY5Y cells. Cells were treated with vanadate (0.1–1 μM) for 24 h in the

presence of 1 mM SIN-1, a peroxynitrite donor, and cell death was assessed using a trypan blue dye exclusion test. SIN-1 caused approximately 40% cell death, and DNA fragmentation was observed by agarose gel electrophoresis, as reported previously [Saeki et al., 2002]. By the addition of the vanadate (1 μM) to the culture medium 30 min before the treatment with SIN-1, cell death was reduced to 10% (Fig. 1A). Similar results were obtained with MTT assay, which measures the level of mitochondrial oxidative phosphorylation (Fig. 1B). When cells were pretreated with vanadate for 30 min followed by removal of it prior to the treatment with SIN-1, the cell death was not prevented. It has been reported that vanadate induces apoptosis [Huang et al., 2000]. Indeed, vanadate at the concentration of 10 μM potentiated the cell death induced by peroxynitrite. This may be due to the vanadate-mediated production of hydroxyl radical from superoxide, as reported previously [Stohs and Bagchi, 1995]. Treatment with okadaic acid (10 nM), an inhibitor of serine/threonine phosphatases 1 and 2A, did not prevent SIN-1-induced cell death. Cyclosporin A (1 μM), an inhibitor of serine/threonine phosphatase 2B, failed to prevent the cell death (Fig. 2).

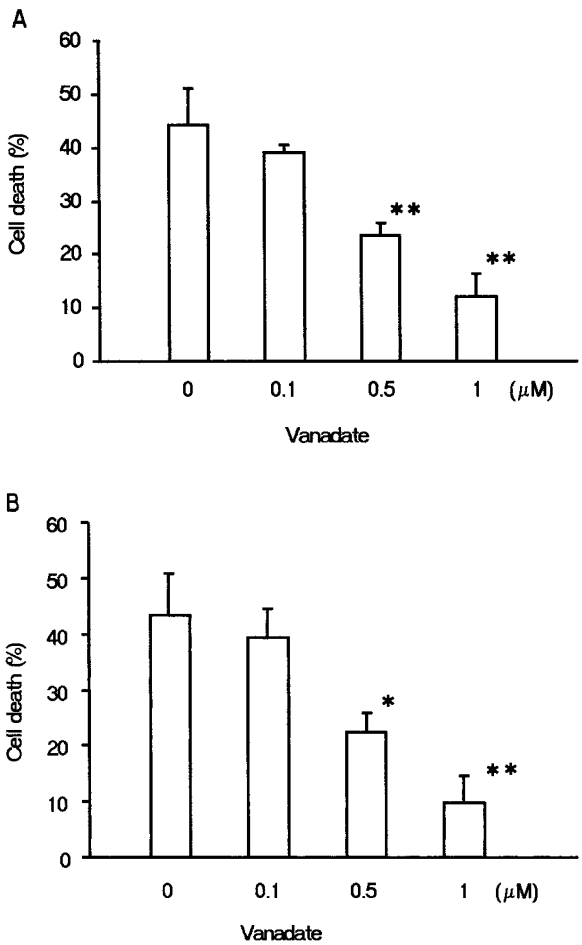

Fig. 1. Protection of SIN-1-induced cell death by vanadate. Indicated concentrations (0.1–1 μM) of vanadate were added to the medium, 30 min before the treatment of SIN-1 (1 mM). Cell viability was evaluated by the failure to exclude trypan blue (**A**) or MTT assay (**B**), 24 h after the SIN-1 treatment. Data are given as means ± SEM from three different experiments. $*P < 0.05$; $**P < 0.01$ compared with that of treatment with SIN-1 alone (0).

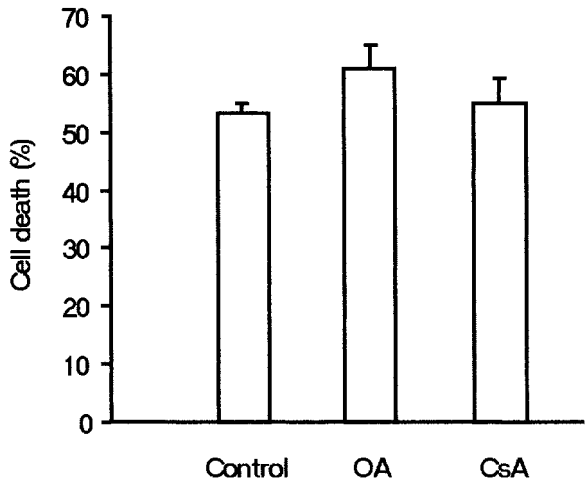

Fig. 2. Effect of serine/threonine phosphatase inhibitor on peroxynitrite-induced cell death. Okadaic acid (OA, 10 nM) or Cyclosporin A (CsA, 1 μM) was added to the medium, 30 min before the treatment of SIN-1 (1 mM). Cell viability was calculated by the failure to exclude trypan blue, 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments.

Protection of Cell Death by Vanadate Is PI3-Kinase-Independent

To determine whether the effect of vanadate involves the PI3-kinase pathway, we tested the effect of wortmannin, an inhibitor of PI3-kinase, on the protection of cell death by vanadate. Wortmannin (100 nM) reversed the protective effect of IGF-1 (10 nM) on SIN-1-induced cell death, but did not reverse that of vanadate (Fig. 3). Similar results were obtained with another inhibitor of PI3-kinase, LY294002.

Vanadate Does not Protect NO-Induced Cell Death

Next, we examined the effect of vanadate on NO-induced cell death. NOC12 (500 μM), a NO donor, caused approximately 50% cell death.

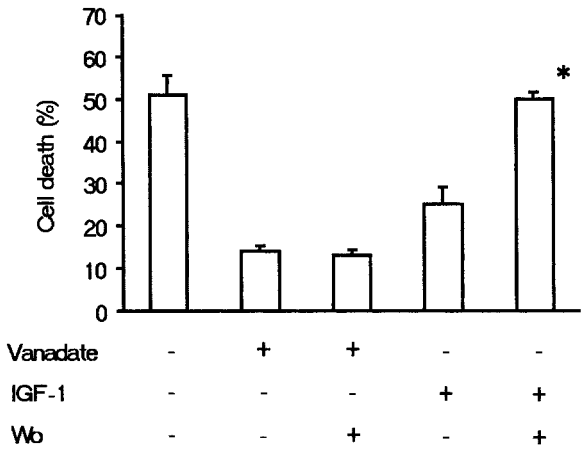

Fig. 3. Effect of PI3-kinase inhibitors on protective effect of vanadate. Vanadate (1 μM) or IGF-1 (10 nM) and wortmannin (100 nM) were added to the medium, 30 min before the SIN-1 treatment (1 mM). Cell viability was calculated by the failure to exclude trypan blue, 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments. $*P < 0.01$ compared with that of treatment with IGF-1.

Vanadate did not prevent NOC12-induced cell death. Neither okadaic acid nor cyclosporin A prevented NOC12-induced cell death (Fig. 4).

Low Concentrations of Vanadate Enhance the Level of Tyrosine Phosphorylation

In order to clarify the mechanism of the protective effect of vanadate on cell death, we investigated tyrosine phosphorylation level using anti-phosphotyrosine immunoblots of lysates from vanadate-treated cells. Vanadate (0.1 μM) treatment for 30 min gave rise to an apparent accumulation of tyrosine phosphorylation of three distinct proteins (Fig. 5). Specifically, tyrosine phosphorylation of the 130-kDa protein was increased by vanadate at the dose which protected cell death. Using specific antibody, we identified the focal adhesion protein $p130^{cas}$ as a component of the 130-kDa protein. We previously reported that tyrosine phosphorylation of $p130^{cas}$ is prevented by nitration induced by peroxynitrite [Saeki and Maeda, 1999]. We examined effects of vanadate on tyrosine phosphorylation of $p130^{cas}$. The extent of tyrosine phosphorylation of $p130^{cas}$ was decreased by SIN-1 treatment, as reported previously [Saeki and Maeda, 1999], and the level of tyrosine phosphorylation of $p130^{cas}$ was recovered by vanadate pretreatment (Fig. 6). NOC12 did not affect the extent of tyrosine phosphorylation of $p130^{cas}$.

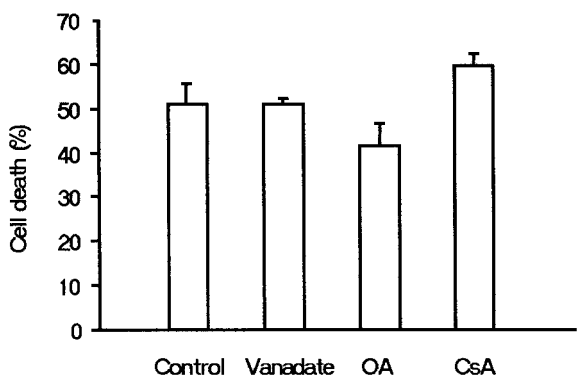

Fig. 4. Effect of phosphatase inhibitor on NOC12-induced cell death. Vanadate (1 μM) was added to the medium, 30 min before the treatment of NOC 12 (500 μM). Cell viability was calculated by the failure to exclude trypan blue, 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments.

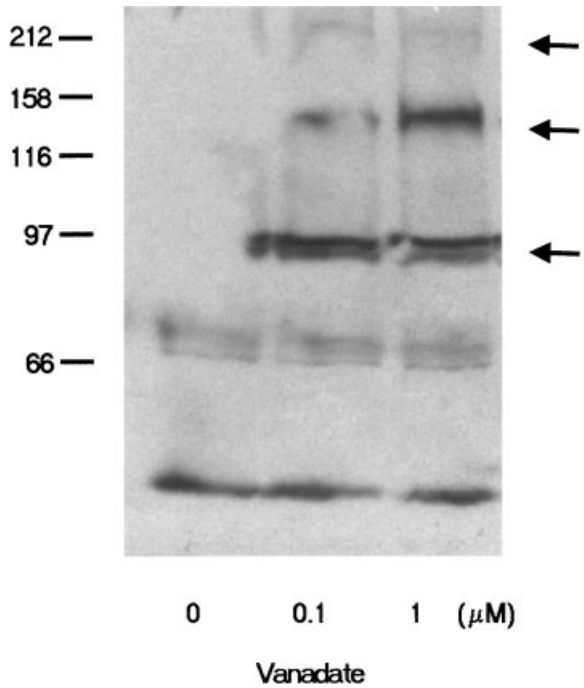

Fig. 5. Tyrosine phosphorylation of proteins induced by vanadate. After the treatment with vanadate at the indicated concentrations for 30 min, whole cell lysates were separated by 7.5% SDS–PAGE and subjected to immunoblotting with anti-phosphotyrosine antibody. Molecular mass markers (in kilodalton) are indicated on the left. Proteins that were tyrosine-phosphorylated as a result of vanadate treatment are indicated by arrows. A given immunoblot is a representative of three independent experiments.

Vanadate Restores the Interaction of $p130^{cas}$ With Crk

Crk is a adaptor molecule containing SH2 domains, which interacts with tyrosine-phos-

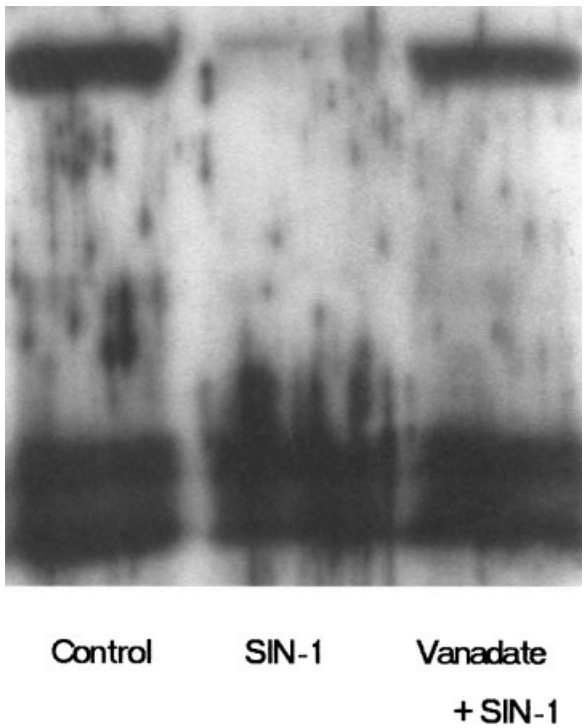

Fig. 6. Tyrosine phosphorylation of p130^{cas} induced by vanadate. Vanadate (1 μM) was added to the medium 30 min before the treatment of SIN-1 (1 mM). Cell lysates were immunoprecipitated with anti-p130^{cas} antibody, separated by 7.5% SDS–PAGE, and subjected to immunoblotting with anti-phosphotyrosine antibody. A given immunoblot is a representative of three independent experiments.

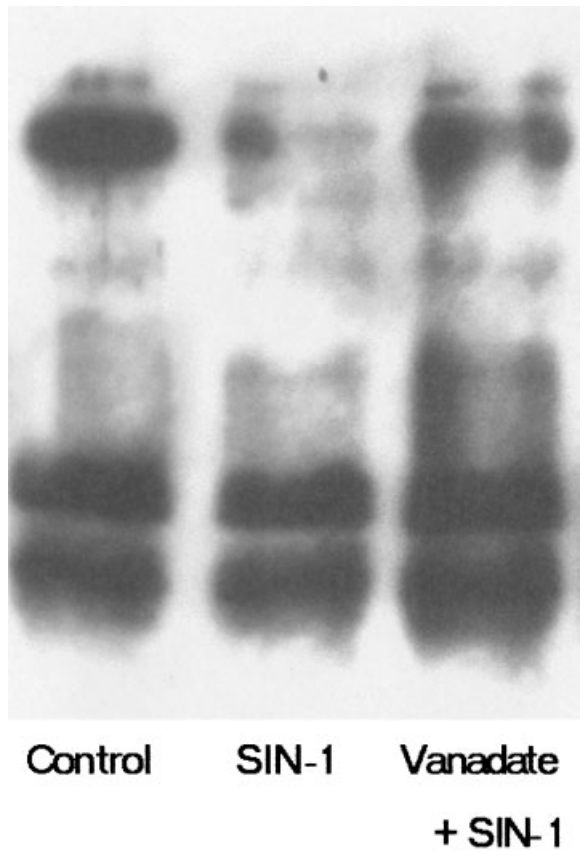

Fig. 7. Effect of vanadate on the interaction between p130^{cas} and Crk. Vanadate (1 μM) was added to the medium 30 min before the treatment of SIN-1 (1 mM). Cell lysates were immunoprecipitated with anti-Crk antibody, separated by 7.5% SDS–PAGE, and subjected to immunoblotting with anti-p130^{cas} antibody. A given immunoblot is a representative of three independent experiments.

phorylated p130^{cas} and promotes cell survival. We investigated the effect of peroxynitrite on the p130^{cas}/Crk interaction. Lysates of cells treated with SIN-1 were immunoprecipitated by anti-Crk antibody and immunoblotted with anti-p130^{cas} antibody. Figure 7 shows that nitration of p130^{cas} reduced the interaction of p130^{cas} with Crk, and that treatment with vanadate restored the effect of nitration. These data suggest that the inhibition of phosphatase may keep p130^{cas} to tyrosine-phosphorylated state, which is able to interact with Crk.

DISCUSSION

In this study, we have shown that vanadate protects peroxynitrite-induced cell death. Protein nitration is a post-translational modification induced by peroxynitrite [Beckman et al., 1994], and the involvement of peroxynitrite-mediated cell death in neurodegenerative disorders, such as Parkinson's disease, is suggested by the localization of nitrotyrosine-like immunoreactivity at sites of tissue damage [Ischiropoulos, 1998]. Nitrotyrosine-containing proteins have been identified in various human and animal diseased tissues [Ischiropoulos, 1998]. We have also shown that the focal adhesion protein p130^{cas} is the target for nitration induced by peroxynitrite [Saeki and Maeda, 1999]. Because nitration of tyrosine residues disrupts tyrosine phosphorylation [Martin et al., 1990; Kong et al., 1996], peroxynitrite might interfere with the signaling mediated by tyrosine kinase. These findings prompted us to explore the effect of tyrosine phosphatase inhibitor on peroxynitrite-induced cell death.

Vanadate has been well documented to mimic many actions of insulin. Although the precise mechanism is not clear, one possibility is that vanadate activates PI3-kinase as suggested in the case of insulin [Band and Posner, 1997]. On

the other hand, it has also been reported that glucose transport stimulation by vanadate is independent of PI3-kinase [Ida et al., 1996; Tsiani et al., 1998]. In this study, the protective effect of vanadate was not prevented by wortmannin, a PI3-kinase inhibitor, suggesting that vanadate protects peroxynitrite-induced cell death through PI3-kinase-independent mechanisms.

The present results do not prove that tyrosine nitration is the direct cause of cell death induced by peroxynitrite. However, it is important to note that vanadate, at a very low concentration, (1 μM) recovered the level of tyrosine phosphorylation and prevented the cell death induced by peroxynitrite. Our results suggest that vanadate modulates the tyrosine phosphorylation of at least three distinct proteins that might be important in peroxynitrite-induced cell death. Although all of these proteins are not identified, the proteins with molecular weight of 130 kDa are proved to include $p130^{cas}$, a focal adhesion protein which is tyrosine-nitrated by peroxynitrite, as reported previously [Saeki and Maeda, 1999]. The protein tyrosine phosphatase PTP-PEST has been shown to interact with and dephosphorylate $p130^{cas}$ [Garton et al., 1996]. Our data are consistent with the report that vanadate inhibits PTP-PEST at low concentrations [Garton et al., 1996].

The role of $p130^{cas}$ in neuronal cell survival is poorly understood, however, recent evidence suggests that $p130^{cas}$ is one of the signaling molecule transmitting cell survival signal induced by cell attachment [Almeida et al., 2000; Cho and Klemke, 2000]. It has been reported that molecular coupling of tyrosine-phosphorylated $p130^{cas}$ and Crk protects against cell death [Cho and Klemke, 2000]. In this study, we have shown that vanadate restores peroxynitrite-induced disruption of the coupling between $p130^{cas}$ and Crk. Although the downstream signals of $p130^{cas}$/Crk coupling are unclear, recent evidence suggests that extracellular-signal-regulated kinase or c-Jun N-terminal kinase is activated by the coupling [Dolfi et al., 1998]. Taken together, we speculate that the protection by vanadate against peroxynitrite-induced cell death might result from enhancing the level of tyrosine phosphorylation of the $p130^{cas}$.

In conclusion, present results suggest that vanadate may keep $p130^{cas}$ to tyrosine-phosphorylated state and allow it to interact with Crk, resulting in activation of the cell survival signaling pathway. In addition, vanadate may provide new approaches to investigate the mechanisms of cell death by nitration.

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