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Antioxidants, vitamin C and dithiothreitol, activate membrane-bound guanylate cyclase in PC12 cells

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Abstract

Antioxidants and antioxidant enzymes are known to protect against cell death induced by reactive oxygen species. However, apart from directly quenching free radicals, little is known about the effect of antioxidants on hormone-activated second messenger systems. We previously found that antioxidants such as 17-β estradiol and resveratrol activate membrane-bound guanylate cyclase GC-A, the receptor for atrial natriuretic factor (ANF), in PC12 cells. It is possible that other antioxidants may also activate membrane-bound guanylate cyclase GC-A. The aim of this study was to determine if dithiothreitol (DTT), vitamin C, and vitamin E activate membrane-bound guanylate cyclase GC-A in PC12 cells. The results showed that both DTT and vitamin C increased cGMP levels in PC12 cells, whereas vitamin E had no effect. DTT and vitamin C inhibited membrane-bound guanylate cyclase activity stimulated by ANF in PC12 cells. In contrast, DTT and vitamin C had no effect on soluble guanylate cyclase activity stimulated by substance P. Furthermore, NO synthase inhibitors L-NAME and aminoguanidine did not affect DTT- and vitamin C-stimulated guanylate cyclase activity. The results indicate that DTT and vitamin C, but not vitamin E, activate membrane-bound guanylate cyclase GC-A in PC12 cells.

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Introduction

Reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hydroxyl radicals have been implicated in ageing and other age-related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease, and Parkinsons disease (Halliwell & Gutteridge 1990; Sagar et al 1992; Ames et al 1993; Sohal & Weindruch 1996). ROS exert damaging effects by reacting with nearly every molecule found in living cells, including DNA, proteins, membrane lipids and carbohydrates. ROS have also been shown to induce apoptosis or necrosis (Buttke & Sandstrom 1994; Jabs et al 1996; Gardner et al 1997). In contrast, addition or overexpression of antioxidants and antioxidant enzymes, such as *N*-acetylcysteine, reduced glutathione, vitamin C, vitamin E, glutathione peroxidase and exogenous catalase, prevents apoptosis in a variety of systems (Malorini et al 1993; Mayer & Noble 1994; Ferrari et al 1995; Jacobson & Raff 1995; Rabizadeh et al 1995). However, little is known about the effect of antioxidants on hormone-activated second messenger systems.

cGMP is a second messenger that mediates the effects of many hormones, such as ATP, serotonin, bradykinin, substance P, and atrial natriuretic factor (ANF) (Murad et al 1988; Drewett & Garbers 1994). There are two types of guanylate cyclase isoforms (membrane-bound and soluble guanylate cyclase) that catalyse the

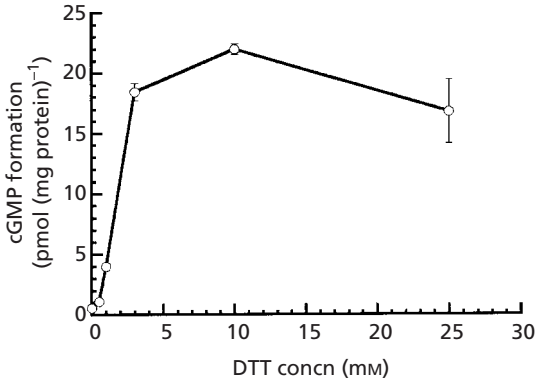

Figure 1 Effects of DTT on cGMP formation in PC12 cells. Confluent cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min and then stimulated with various doses of DTT for a further 10 min. The reaction was stopped with 10% trichloroacetic acid. Generated cGMP was measured by radioimmunoassay. DTT stimulated cGMP formation in a dose-dependent manner with an EC50 at approximately 3 mM. Data represent mean ± s.d. of four replicates.

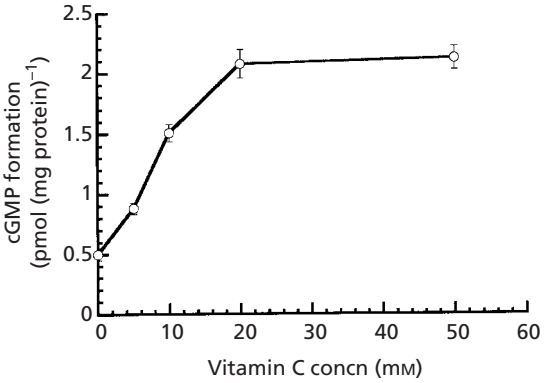

Figure 2 Effects of vitamin C on cGMP formation in PC12 cells. Confluent cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min, and then stimulated with various doses of vitamin C for a further 10 min. The reaction was stopped with 10% trichloroacetic acid. Generated cGMP was measured by radioimmunoassay. Vitamin C increased cGMP levels in a dose-dependent manner with an EC50 at approximately 7 mM. Data represent mean ± s.d. of four replicates.

formation of cGMP from GTP. Membrane-bound guanylate cyclase GC-A is the receptor for ANF and activated by ANF, whereas soluble guanylate cyclase is activated by NO generated by NO synthase or NO donors (Murad et al 1988; Drewett & Garbers 1994). Recently, we found that antioxidants such as 17-β estradiol and resveratrol activate membrane-bound guanylate cyclase GC-A in PC12 cells (Chen et al 1998, 2000). To determine if other antioxidants also affect the ANF/membrane-bound guanylate cyclase system or substance P/soluble guanylate cyclase system, we measured the effect of dithiothreitol (DTT), vitamin C, and vitamin E on basal, ANF-stimulated and substance P-stimulated cGMP formation in PC12 cells.

Materials and Methods

Materials

Synthetic rat ANF (8–33) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Vitamin C, vitamin E, DTT and other common chemicals were purchased from Sigma (St Louis, MO).

cGMP determination

PC12 cells were cultured until confluence in 6-well plates (35 mm) with RPMI medium containing 5% bovine fetal serum and 10% horse serum. The cells were washed with 2 mL serum-free medium and preincubated at 37°C for 10 min with 900 μL of medium containing 0.5 mM isobutylmethylxanthine. Various concentrations of DTT, vitamin C and vitamin E were added to the cells and incubated for 10 min at 37°C. After incubation, the medium was aspirated and 1 mL cold 10% trichloroacetic acid was added to the plates. The cell extracts were scraped and centrifuged for 15 min at 2000 *g*, and the supernatant fractions were extracted with water-saturated ether to remove trichloroacetic acid. The cGMP levels in the supernatants were determined by radioimmunoassay (Chang et al 1990; Chang & Song 1993; Miao et al 1995; Chen et al 1998, 2000).

Results

Effects of DTT, vitamin C and vitamin E on cGMP formation in PC12 cells

To determine if antioxidants affect guanylate cyclase activity, we measured the effects of DTT and vitamin C on cGMP formation in PC12 cells. The results indicate that DTT (Figure 1) and vitamin C (Figure 2) increased cGMP levels in PC12 cells in a dose-dependent manner. The maximum activation for DTT and vitamin C was approximately 44- and 4.25-fold, respectively. The EC50 values for activation of guanylate cyclase activity by DTT and vitamin C were 3 and 7 mM, respectively.

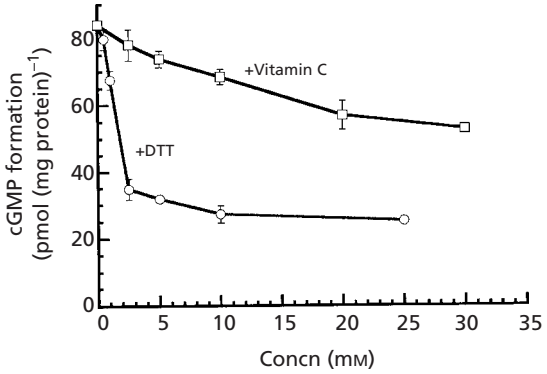

Figure 3 DTT and vitamin C inhibited ANF-stimulated guanylate cyclase activity in PC12 cells. Confluent cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min, and stimulated with various doses of DTT or vitamin C for 10 min and then stimulated with 0.1 μM ANF for a further 10 min. The reaction was stopped with 10% trichloroacetic acid. Generated cGMP was measured by radioimmunoassay. Both DTT and vitamin C inhibited ANF-stimulated guanylate cyclase activity. Data represent mean $\pm$ s.d. of four replicates.

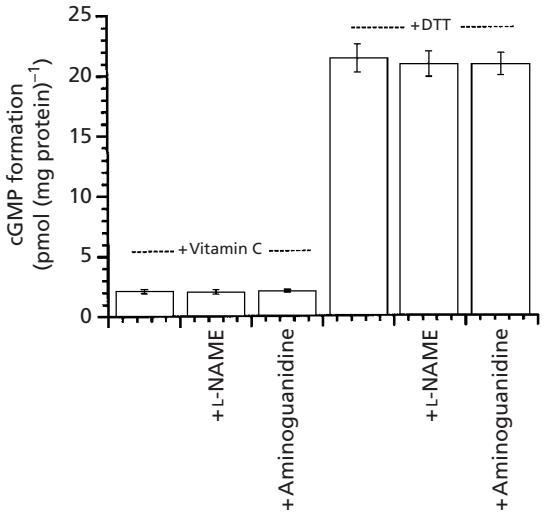

Figure 4 Effects of L-NAME and aminoguanidine on DTT- and vitamin C-stimulated guanylate cyclase activity in PC12 cells. PC12 cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min, preincubated with L-NAME (2 μM) or aminoguanidine (600 μM) for 10 min and then challenged with 10 mM DTT or 20 mM vitamin C for a further 10 min. The reaction was stopped with 10% trichloroacetic acid. The generated cGMP was measured by radioimmunoassay. Neither L-NAME or aminoguanidine affected DTT-stimulated and vitamin C-stimulated guanylate cyclase activity. Data represent mean $\pm$ s.d. of four replicates.

Vitamin E had no effect on cGMP formation (data not shown).

Effects of DTT and vitamin C on ANF- and substance P-stimulated guanylate cyclase activity in PC12 cells

There are two general types of guanylate cyclase isoforms present in the cells, soluble and membrane-bound guanylate cyclase (Murad et al 1988; Drewett & Garbers 1994). Membrane-bound guanylate cyclase GC-A is the receptor for ANF. Binding of ANF to GC-A stimulates the enzyme activity. Soluble guanylate cyclase is activated by hormones such as ATP, oxytocin, and substance P through NO. To determine which guanylate cyclase isoform is activated by DTT and vitamin C, we measured the effects of DTT and vitamin C on 0.1 μM ANF- and 1 μM substance P-stimulated guanylate cyclase activity in PC12 cells. Figure 3 shows that both DTT and vitamin C inhibited ANF-stimulated cGMP formation in a dose-dependent manner. In contrast, DTT and vitamin C had no effect on substance P-stimulated cGMP formation (data not shown). Furthermore, the NO-synthase inhibitors, L-NAME and aminoguanidine, had no effect on 10 mM DTT- and 20 mM vitamin C-stimulated cGMP formation (Figure 4). These results suggest that DTT and vitamin C increase cGMP levels through the activation of the ANF/membrane-bound guanylate cyclase pathway, but not the NO/soluble guanylate cyclase pathway.

Discussion

Recently, we found that 17-β estradiol and resveratrol activate membrane-bound guanylate cyclase GC-A in PC12 cells (Chen et al 1998, 2000). Since 17-β estradiol and resveratrol also exert antioxidant effects, it is possible that other antioxidants may activate the ANF/membrane-bound guanylate cyclase system. To examine this possibility, we determined the effects of DTT, vitamin C and vitamin E on basal and ANF-stimulated guanylate cyclase activity in PC12 cells. The results indicate that DTT and vitamin C increase cGMP formation in PC12 cells, whereas vitamin E has no effect on guanylate cyclase activity. The EC50 for activation of guanylate cyclase activity by vitamin C was 7 mM. The concentration of vitamin C in the plasma is approximately 50 μM, 10-fold higher in cerebrospinal fluid, up to 1 mM in glia, and 10 mM in neurons (Specter 1977; Milby 1982; Rice 2000). It should be noted that these reported concentrations of vitamin C represent steady-state levels in tissues. Local fluctuations in vitamin C levels in tissues may exist. Thus, it may be possible that the physiological concentration of vitamin C in tissues, particularly in neurons, may significantly stimulate the

ANF/membrane-bound guanylate cyclase pathway. The concentrations of DTT used in many reports are over the range of 1–100 mM. For instance, treatment of cells with 2 mM DTT prevents TGF-β binding to its type 1 receptor (Wells et al 1999). DTT at a concentration of 5 mM significantly reduces the angiotensin II-induced contractile response in the rabbit aorta (Kawano at al 1998). At a concentration of 10 mM, DTT reverses the inhibitory effect of orthovanadate on calcineurin activity (Morioka et al 1998) and inhibits fetal cord serum ultrafiltrate-induced sperm capacitation and superoxide production (de Lamirande & Gagnon 1998). DTT at a concentration of 100 mM has been used to reverse the inhibition of H^+-ATPase activity by S-nitrosoglutathione (Forgac 1999). In this study, we found that the EC50 for the activation of guanylate cyclase by DTT was 3 mM.

Several lines of investigation indicate that vitamin C and DTT increase cGMP formation through the activation of the ANF receptor/membrane-bound guanylate cyclase pathway, but not the NO/soluble guanylate cyclase pathway. Firstly, vitamin C and DTT do not affect soluble guanylate cyclase activity stimulated by substance P. Secondly, NO synthase inhibitors have no effect on vitamin C- and DTT-stimulated cGMP formation, indicating that DTT and vitamin C do not increase cGMP through NO/soluble guanylate cyclase signalling pathway. Thirdly, vitamin C and DTT reduce ANF-stimulated, but not substance P-stimulated, guanylate cyclase activity. Both vitamin C and DTT are less potent than ANF in stimulating guanylate cyclase activity. Therefore, when vitamin C and DTT are added together with ANF, they compete with ANF for the activation of guanylate cyclase and thus lower ANF-stimulated guanylate cyclase activity in PC12 cells. These results demonstrate that vitamin C and DTT can also activate the second messenger system in addition to detoxifying the action of ROS.

Several clinical studies have shown that plasma vitamin C levels are inversely related to systolic and diastolic pressure (Yoshioka et al 1984; Bulpitt 1990; Moran et al 1993; Ness et al 1996), and that vitamin C lowers blood pressure in hypertensive patients (Duffy et al 1999). However, the mechanisms by which vitamin C regulates blood pressure remain unclear. Moran et al (1993) suggest that vitamin C may prevent the oxidation of lipids and may therefore have an antihypertensive effect. Koh (1984) proposes that vitamin C prevents hypertension by lowering the sodium content in the blood. Trout (1991) suggests that plasma vitamin C may lower blood pressure in part by altering leukotriene metabolism. Recently, Taddei et al (1998) showed that vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertensive patients. The ANF/membrane-bound guanylate cyclase signalling pathway is also involved in blood pressure regulation because ANF (John et al 1995) and membrane-bound guanylate cyclase GC-A (Lopez et al 1995) knockout mice are hypertensive, and transgenic mice overexpressing ANF are hypotensive (Steinhelper et al 1990). Therefore, it is possible that the hypotensive effect of vitamin C may be partly due to the activation of membrane-guanylate cyclase.

In conclusion, the results demonstrate that the antioxidants vitamin C and DTT activate membrane-bound guanylate cyclase GC-A in PC12 cells. Since membrane-bound guanylate cyclase GC-A is the receptor for ANF, these results indicate that antioxidants can also exert their effects by interacting with other receptors.

References

Ames, B., Shigenaga, M. K., Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl Acad. Sci. USA* **90**: 7915–7922

Bulpitt, C. J. (1990) Vitamin C and blood pressure. *J. Hypertens.* **8**: 1071–1075

Buttke, T. M., Sandstrom, P. A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today* **15**: 7–10

Chang, C.-H., Song, D.-L. (1993) Melittin potentiates guanylate cyclase activation stimulated by atrial natriuretic factor and ATP. *J. Biol. Chem.* **268**: 4908–4911

Chang, C.-H., Kohse, K. P., Chang, B, Hirata, M., Jiang, B., Douglas, J., Murad, F. (1990) Characterization of ATP-stimulated guanylate cyclase activation in rat lung membranes. *Biochim. Biophys. Acta* **1052**: 159–165

Chen, Z.-J., Yu, L., Chang, C.-H. (1998) Stimulation of membrane-bound guanylate cyclase activity by 17-ß estradiol. *Biochem. Biophys. Res. Commun.* **252**: 639–642

Chen, Z.-J., Che, D., Chang, C.-H. (2000) Resveratrol activates membrane-bound guanylate cyclase in PC12 cells. *Pharm. Pharmacol. Commun.* **6**: 211–215

de Lamirande, E., Gagnon, C. (1998) Paradoxical effect of reagents for sulfhydryl and disulfide groups on human sperm capacitation and superoxide production. *Free Radic. Biol. Med.* **25**: 803–817

Drewett, J. G., Garbers, D. L. (1994) The family of guanylyl cyclase receptors and their ligands. *Endocr. Rev.* **15**: 135–162

Duffy, S. J., Gokce, N., Holbrook, M., Huang, A., Frei, B., Keaney, J. F., Vita, J. A. (1999) Treatment of hypertension with ascorbic acid. *Lancet* **354**: 2048–2049

Ferrari, R, Yan, C. Y., Greene, L. A. (1995) N-acetylcysteine (D- and L-isomers) prevents apoptotic death of neuronal cells. *J. Neurosci.* **15**: 2857–2866

Forgac, M. (1999) The vacuolar H^+-ATPase of clathrin-coated vesicles is reversibly inhibited by S-nitrosoglutathione. *J. Biol. Chem.* **274**: 1301–1305

Gardner, A. M., Xu, F. H., Fady, C., Jacoby, F. J., Duffey, D. C., Tu, Y., Lichtenstein, A. (1997) Apoptotic vs non-apoptotic cytotoxicity induced by hydrogen peroxide. *Free Radic. Biol. Med.* **22**: 73–83

Halliwell, B., Gutteridge, M. C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **186**: 1–85

Jabs, T., Dietrich, R. A., Dangl, J. L. (1996) Initiation of runaway cell death in an arabidopsis mutant by extracellular superoxide. *Science* **273**: 1853–1856

Jacobson, M. D., Raff, M. C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374**: 814–816

John, S. W. M., Krege, J. H., Oliver, P. M., Hagaman, J. R., Hodgin, J. B., Pang, S. C., Flynn, T. G., Smithies, O. (1995) Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science* **267**: 679–681

Kawano, K., Fujishima, K., Nagura, J., Yasuda, S., Shinki, Hachisu, M., Konno, F. (1998) Nonpeptide angiotensin II receptor antagonist recognizes inter-species differences in angiotensin AT1 receptors. *Eur. J. Pharmacol.* **357**: 33–39

Koh, E. T. (1984) Effects of vitamin C on blood parameters of hypertensive subjects. *J. Okla. State Med. Assoc.* **77**: 177–182

Lopez, M. J., Wong, S. K.-F., Kishimoto, I., Dubois, S., Mach, V., Friesen, J., Garbers, D. L., Beuve, A. (1995) Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. *Nature* **378**: 65–68

Malorini, W., Rivaben, R., Santini, M. T., Donelli, G. (1993) N-acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. *FEBS Lett.* **327**: 75–78

Mayer, M., Noble, M. (1994) N-acetylcysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. *Proc. Natl Acad. Sci. USA* **91**: 7496–7500

Miao, Z.-H, Song, D.-L, Douglas, G. D., Chang, C.-H. (1995) Mutational inactivation of the catalytic domain of guanylate cyclase-A receptor. *Hypertension* **25**: 694–698

Milby, K. (1982) Detailed mapping of ascorbate distribution in rat brain. *Neurosci. Lett.* **28**: 15–20

Moran, J. P., Cohen, L., Greene, J. M., Xu, G., Feldman, E. B., Hames, C. G., Feldman, D. S. (1993) Plasma ascorbic acid concentrations relate inversely to blood pressure in human subjects. *Am. J. Clin. Nutr.* **57**: 213–217

Morioka, M., Fukunaga, K., Kawano, T., Hasegawa, S., Korematsu, K., Kai, Y., Hamada, J., Miyamoto, E., Ushio, Y. (1998) Serine/threonine phosphatase activity of calcineurin is inhibited by sodium orthovanadate and dithiothreitol reverses the inhibitory effect. *Biochem. Biophys. Res. Commun.* **253**: 342–345

Murad, F., Leitman, D., Waldman, S., Chang, C.-H., Hirata, M., Kohse, K. (1988) Effects of nitrovasodilators, endothelium-dependent vasodilators and atrial peptides on cyclic GMP. *Cold Spring Harb. Symp. Quant. Biol.* **53**: 1005–1009

Ness, A. R., Khaw, K. T., Bingham, S., Day, N. E. (1996) Vitamin C status and blood pressure. *J. Hypertens.* **14**: 503–508

Rabizadeh, S., Gralla, E., Borchelt, D., Gwinn, R., Valentine, J., Sisodia, S., Wong, P., Lee, M., Hahn, H., Bredesen, D. (1995) Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: studies in yeast and neural cells. *Proc. Natl Acad. Sci. USA* **92**: 3024–3028

Rice, M. E. (2000) Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci.* **23**: 209–216

Sagar, S., Kallo, J. I., Kaul, N., Granguly, N. K., Sharma, B. K. (1992) Oxygen free radicals in essential hypertension. *Mol. Cell Biochem.* **111**: 103–108

Specter, R. (1977) Vitamin homeostasis in the central nervous system. *N. Engl. J. Med.* **296**: 1393–1398

Sohal, R. S., Weindruch, R. (1996) Oxidative stress, caloric restriction, and aging. *Science* **273**: 59–63

Steinhelper, M. E., Cochrane, K. L., Field, L. J. (1990) Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension* **16**: 301–307

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., Salvetti, A. (1998) Vitamin C improves endothelium-dependent vasodilation by restoring nitric acid activity in essential hypertension. *Circulation* **97**: 2222–2229

Trout, D. L. (1991) Vitamin C and cardiovascular risk factors. *Am. J. Clin. Nutr.* **53**: 322S–325S

Wells, R. G., Gilboa, L., Sun, Y., Liu, X., Henis, Y., Lodish, H. F. (1999) Transforming growth factor-β induces formation of a dithiothreitol-resistant type I/type II receptor complex in live cells. *J. Biol. Chem.* **274**: 5716–5722

Yoshioka, M, Matsushita, T, Chuman, Y. (1984) Inverse association of serum ascorbic acid level and blood pressure or rate of hypertension in male adults aged 30–39 years. *Int. J. Vitam. Nutr. Res.* **54**: 343–347