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Short communication

Nitric oxide scavenger carboxy-PTIO potentiates the inhibition of dopamine uptake by nitric oxide donors

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Abstract

2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) has been increasingly used as nitric oxide (NO) scavenger. Carboxy-PTIO reacts with NO to form nitric dioxide and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI). In rat C6 glioma cells expressing human dopamine transporter, carboxy-PTIO paradoxically potentiated the inhibition of [^{3}H]dopamine uptake by two NO donors, diethylamine/NO and (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)-amino]/NO. Further examinations revealed that carboxy-PTI concentration-dependently reduced dopamine uptake, indicating that the formation of carboxy-PTI may account for the failure of carboxy-PTIO to abolish NO elicited effects. These results suggest that caution should be taken in interpreting data obtained using carboxy-PTIO and probably other NO scavengers.

Keywords: Dopamine uptake; Nitric oxide (NO) scavenger; Carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide); Carboxy-PTI (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl)

1. Introduction

During the last decade, there has been an explosion in interest for understanding the involvement of nitric oxide (NO) in a wide variety of both physiological and pathophysiological processes. To demonstrate that a biological response is mediated by this simple free radical gas, various compounds that could inactivate NO have been used to prevent the effects of NO (Feelisch, 1998). In large part, the use of the so-called NO scavengers has developed as a complement to the application of NO donors, rather than authentic NO, in most studies of NO-related biological effects. The nitronyl nitroxides, which were introduced earlier for the quantification of NO by electron spin resonance technique, inactivate NO by oxidative transformation to nitric dioxide (NO_2) and imino nitroxides (Akaike et al., 1993; Az-ma et al., 1994; Hogg et al., 1995; Konorev et al., 1995). Among compounds of this class, phenyl-tetramethylimidazolineoxyl-oxides (PTIO) and especially its water-soluble derivative carboxy-PTIO have become very popular NO scavengers and have been increasingly used in the field of NO related studies, although the specificity of carboxy-PTIO as NO scavenger was once questioned (Pfeiffer et al., 1997).

Using rat C6 glioma cells transfected with human dopamine transporter, we characterized the effect of various NO generators on dopamine uptake, and we observed a correlation between NO generating capability and inhibition of dopamine uptake (Cao and Reith, manuscript in preparation). Based on these results and similar observations in other dopamine uptake systems by others (Lonart and Johnson, 1994; Pogun et al., 1994), we expected that carboxy-PTIO would prevent the reduction of dopamine uptake by NO generating drugs in C6 cells expressing the human dopamine transporter. Surprisingly, we found that carboxy-PTIO actually potentiated the effects of diazeniumdiolates diethylamine/NO and (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)-amino]/NO (PAPA/NO), which spontaneously generate NO when dissolved in aqueous media (Fitzhugh and Keefer, 2000). To assess whether the failure of carboxy-PTIO to attenuate the effect of NO donors was due to the formation of the reaction products, we undertook additional experiments to compare the effects of carboxy-PTIO with that of its reaction product 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI). The results showed that, while carboxy-PTIO reduces dopamine accu-

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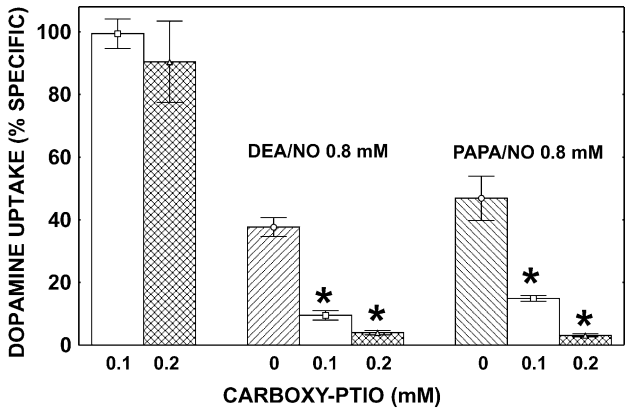

Fig. 1. Potentiation of the effect of diethylamine (DEA)/NO and PAPA/NO on dopamine uptake by nitric oxide scavenger carboxy-PTIO in rat C6 glioma cells expressing human dopamine transporter. Cells were preincubated with drugs at 21 °C for 15 min. The amount of [^{3}H] dopamine in vehicle-treated wells was approximately 13,800 cpm. All drugs were tested with triplicate determinations in three independent experiments. * Compared with corresponding control values (DEA/NO or PAPA/NO alone), $P < 0.01$.

mulation at higher concentrations, the inhibitory action of carboxy-PTI is much more potent.

2. Materials and methods

2.1. Chemicals

[^{3}H]Dopamine (54.8 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Diethylamine/NO, PAPA/NO and carboxy-PTIO were obtained from Alexis Chemical (San Diego, CA, USA). Carboxy-PTI was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Rat C6 glioma cells stably expressing human dopamine transporter

The cloning of a human dopamine transporter cDNA and its transfection into C6 glioma cells were performed in the laboratory of Dr. Aaron Janowsky at Oregon Health Sciences University, Portland, OR (Eshleman et al., 1995). Cells stably expressing the human dopamine transporter were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% bovine calf serum and 1 μg/ml puromycin. Before experiments, C6 cells were seeded onto and further cultured in 96-well plates.

2.3. [^{3}H]Dopamine uptake

The medium was removed. Cells were washed twice with wash buffer (122 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 15 mM Na_2HPO_4) and then preincubated with assay buffer (identical to wash buffer plus 0.1 mM tropolone) and appropriate drugs at room temperature (21 °C) for 15 min. Uptake was initiated by the addition of 20 nM [^{3}H]dopamine. The final assay volume was 250 μl. Cocaine (0.1 mM) was used to define non-specific uptake. Following an 8-min incubation, assays were terminated by removing the medium, after which the plate was placed on ice and each well was washed twice with 250 ml of ice-cold phosphate-buffered saline. Trichloroacetic acid (3%) was added to each well, and the radioactivity was determined by conventional liquid scintillation spectrometry.

2.4. Data analyses

Results are expressed as mean ± S.E. Comparisons were made by analysis of variance (ANOVA) followed by Duncan Multiple Comparison Test.

3. Results

3.1. NO scavenger carboxy-PTIO does not antagonize, but potentiates NO-induced inhibition of dopamine uptake

As established before (Cao and Reith, unpublished data), diethylamine/NO and PAPA/NO with spontaneous release NO in the assay buffer decreased the accumulation of [^{3}H]dopamine in rat C6 glioma cells transfected with human dopamine transporter. Exposure of the cells to diethylamine/NO or PAPA/NO (0.8 mM) caused decreases in dopamine uptake by 62% and 53%, respectively (Fig. 1). At concentrations of 0.1 or 0.2 mM, carboxy-PTIO itself was without significant effect on dopamine uptake but was able to significantly potentiate the inhibition caused by diethylamine/NO and PAPA/NO ($P < 0.01$). Thus, when diethylamine/NO was co-incubated with carboxy-PTIO, a significantly increased inhibition of dopamine uptake was observed. In cells exposed to 0.1 or 0.2 mM carboxy-PTIO

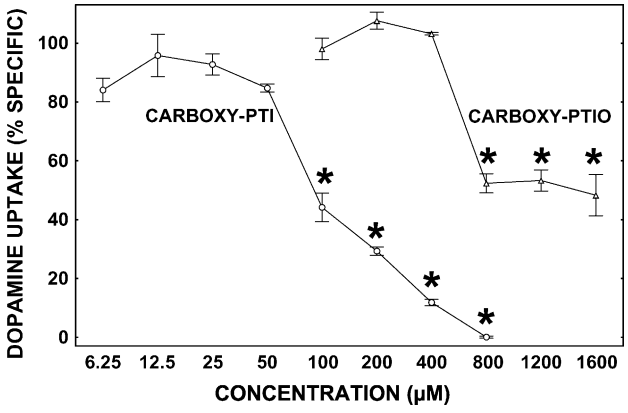

Fig. 2. Comparison of the effects of carboxy-PTIO and carboxy-PTI on dopamine uptake. The amount of [^{3}H]dopamine in vehicle-treated wells was approximately 14,800 cpm. Drugs were tested with triplicate determinations in three independent experiments. * Compared with vehicle-treated cells, $P < 0.01$.

and 0.8 mM diethylamine/NO, the reduction of dopamine uptake reached 90% and 96%, respectively. Carboxy-PTIO potentiated PAPA/NO-induced inhibition to a similar extent.

3.2. Carboxy-PTI decreases dopamine uptake

The effects of carboxy-PTIO and its reaction product with NO, carboxy-PTI, were further examined. At concentrations from 0.1 to 0.4 mM, carboxy-PTIO had no influence on [^{3}H]dopamine uptake. At 0.8 mM, this compound caused about 50% reduction in dopamine uptake. Increasing concentration would not lead to further inhibition. Unlike carboxy-PTIO, the inhibition of dopamine uptake by carboxy-PTI was fully concentration-dependent. At 0.8 mM, the inhibition was almost complete (Fig. 2).

4. Discussion

It is a common practice to employ NO scavengers to demonstrate the specificity of NO effects. If a given biological response is not prevented or reversed by a NO scavenger, it is generally concluded that this response is not mediated by NO. Carboxy-PTIO is a potent NO scavenger and has been used in vivo to inhibit hypotension and endotoxic shock induced by lipopolysaccharide which increases NO synthesis (Yoshida et al., 1994) and in vitro to prevent NO-induced vascular relaxation (Akaike et al., 1993; Konorev et al., 1995; Pieper and Siebeneich, 1997; Rand and Li, 1995). The effects of carboxy-PTI, a reaction product of carboxy-PTIO and NO, has been occasionally reported. For example, this compound causes vasodilatation in the canine coronary arteries (Tsunoda et al., 1994). Unfortunately, little attention has been paid as to whether the biological activity of carboxy-PTI will interfere its use of its parent compound carboxy-PTIO.

We initially wanted to use NO scavengers to demonstrate that the released NO is responsible for the reduction in dopamine uptake induced by NO donors in rat C6 glioma cells expressing human dopamine transporter. Although another NO scavenger hydroxocobalamin could completely abolish the inhibitory effect of diethylamine/NO and PAPA/NO (Cao and Reith, unpublished data), carboxy-PTIO could not reverse the effect of these two NO donors. Furthermore, at concentrations not altering the functions of dopamine transporter, carboxy-PTIO further potentiated the inhibition produced by diethylamine/NO and PAPA/NO. This unexpected finding prompted us to examine the effects of reaction products of carboxy-PTIO and NO. Compared with carboxy-PTIO and NO generating agents diethylamine/NO and PAPA/NO, carboxy-PTI was more potent in reducing dopamine uptake. It is therefore reasonable to assume that the formation of carboxy-PTI at least partially account for the failure of carboxy-PTIO to prevent NO donors' effect on dopamine uptake. It is not clear whether nitric dioxide, another reaction product of carboxy-PTIO and NO, affects dopamine uptake. It was recently reported that, while carboxy-PTI does not exert any action on prostacyclin synthase, the prostacyclin synthase inactivating effect of carboxy-PTIO is mediated by NO_2 (Soler et al., 2001). At this time, the mechanism by which carboxy-PTI exerts its inhibitory effect on dopamine uptake remains unknown. This compound did not alter the binding of the cocaine analog (2β-carbomethoxy-3β-(4-fluorophenyl)[^{3}H]tropane ([^{3}H]WIN 35,428) (Cao and Reith, unpublished observation).

The lack of attenuation on the effects elicited by NO releasing agents has been previously observed with carboxy-PTIO and has been interpreted as non-involvement of NO in the corresponding biological process (Mitsutomi et al., 1999; Nomura et al., 1998). Imam et al. (2000) reported that the NO synthase inhibitor 7-nitroindazole could diminish the neurotoxicity of methamphetamine in mice, but carboxy-PTIO could not. This is unexpected because generation of carboxy-PTI, if sufficient, could have blocked dopamine uptake and it is known that dopamine uptake inhibition blocks the long-term effect of metamphetamine (see Fumagalli et al., 1998).

Our results may have implications for better understanding the mode of action of carboxy-PTIO and other related compounds as NO scavengers in studies on dopamine transport and possibly other biological systems. Although carboxy-PTIO can scavenge NO, the formation of carboxy-PTI and/or nitric dioxide may complicate the interpretation of data if carboxy-PTI possesses activity similar to that of NO as is the case for inhibiting dopamine transport. Therefore, the failure of carboxy-PTIO (any possibly other NO scavengers) to attenuate the effects of NO would not naturally lead to the conclusion of "no NO involvement." While an NO scavenger such as carboxy-PTIO could attenuate the NO elicited effects as expected, the possibility that its reaction product(s) contribute to the antagonism should be explored. In order to avoid misinterpretation, we would suggest that both carboxy-PTI and carboxy-PTIO should be included as controls by themselves.

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