Early Suppression of Striatal Cyclic GMP May Pre-Determine the Induction and Severity of Chronic Haloperidol-Induced Vacuous Chewing Movements

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Received: 16 December 2000; Accepted: 15 January 2001

Haloperidol persists in brain tissue long after discontinuation while haloperidol -induced tardive dyskinesia often worsens after withdrawal of the drug. The mechanism of haloperidol -associated tardive dyskinesia is unknown, although neurotoxic pathways are suspected. Nitric oxide (NO) synthase (NOS) inhibitors exacerbate haloperidol -induced catalepsy, while haloperidol itself is a potent neuronal NOS inhibitor in vitro. Since NO and cGMP are involved in striatal neural plasticity, this study investigates a possible relation between cGMP and extrapyramidal symptoms as early predictors of haloperidol -associated tardive dyskinesia. Sprague-Dawley rats were administered either water or oral haloperidol (0.25mg/kg/d po) for 17 weeks, followed by 3 weeks withdrawal. Saline (ip) or the nNOS/guanylate cyclase inhibitor, methylene blue (5mg/kg/d ip), were co-administered with haloperidol for the first three weeks of treatment. Vacuous chewing movements (VCM's) were continuously monitored, followed by the determination of striatal cGMP and peripheral serum nitrogen oxide (NOx) levels. Chronic haloperidol engendered significant VCM's, with acute withdrawal associated with significantly reduced striatal cGMP levels as well as reduced serum NOx. Furthermore, suppressed cGMP levels were maintained and VCM's were significantly worse after early administration of methylene blue to the chronic haloperidol group. However, serum NOx was unchanged from control. We conclude that the central effects of chronic haloperidol on striatal NO-cGMP function persist for up to 3 weeks post-withdrawal. Moreover, suppression of striatal cGMP constitutes an early neuronal insult that determines the presence and intensity of haloperidol -associated motor dysfunction.

Key words: Haloperidol, extrapyramidal symptoms, tardive dyskinesia, nitric oxide, cGMP, methylene blue.

INTRODUCTION

The neurochemical basis for haloperidol-induced tardive dyskinesia is unknown. It is, however, well known that in patients receiving high-dose haloperidol over an extended period and where chronic, uncontrolled early-stage extrapyramidal symptoms accompanies the treatment, there is an increased risk for the development of this potentially irreversible

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syndrome (Harvey et al., 1999). These observations have suggested a link between haloperidol-associated tardive dyskinesia and the duration and intensity of early-stage extrapyramidal symptoms. The irreversible nature of tardive dyskinesia hints at a neurotoxic component, possibly resulting in some degree of permanent brain damage (APA Task force, 1992). This is supported by evidence that patients with tardive dyskinesia display increased activity of the excitotoxic neurotransmitter, glutamate (Tsai et al., 1998), while haloperidol also induces oxidative radical-based toxicity to neuronal cells in culture (Behl et al., 1996). Moreover, long-term administration of haloperidol to rats is also associated with an increase in striatal hydrogen peroxide and lipid radical formation in vivo (Yokoyama et al., 1998).

Haloperidol displays an elimination half-life in human brain tissue of 6.8 days (Kornhuber et al., 1999), while the serum elimination half-life of the drug in humans slows with time after administration and may be measured in days rather than hours (Hubbard et al., 1987). A comparable effect has been observed in experimental animals (Cohen et al., 1992). Haloperidol is bio-transformed to a neurotoxic pyridinium species known as HPP+ that is toxic to dopaminergic neurons through inhibition of site 1 of the mitochondrial electron transport chain (Avent et al., 1996). Thus, the pharmacokinetic characteristics of haloperidol, coupled with its ability to induce cell toxic sub-cellular events, may suggest a mechanism whereby the drug may induce long-term neurotoxicity, even after the drug has been withdrawn.

Much clinical and experimental evidence has accumulated in support of a role for nitric oxide (NO) in the pathogenesis of a number of neuro-psychiatric disorders, including Parkinson's disease, stroke, Alzheimer's disease, schizophrenia and depression (Harvey, 1996). Apart from its acknowledged role as an inter- and intrain-cellular messenger and neurotransmitter (Harvey, 1996), recent attention has focused on its potential neurotoxic actions via the generation of reactive nitrogen species such as peroxynitrite (ONOO-) and nitrogen dioxide radicals (Lipton et al., 1993). However, NO is both a reducing as well as an oxidizing agent (Lipton et al., 1993). This attribute affords it with both neurotoxic and neuroprotective properties. Indeed, NO has been found to reduce the generation of reactive oxygen species, such as hydrogen peroxide and superoxide, and to prevent lipid peroxidation (Kanner et al., 1992). Thus, despite the formation of various reactive nitrogen species by NO, NO can also protect against oxidative stress mediated by reactive oxygen species (Wink et al., 1999) and exercise a protective effect on oxidative tissue damage. Both NO as well as its principle second messenger, cyclic guanosine monophosphate (cGMP), are also involved in cellular memory, especially the regulation of neuronal plasticity and long-term potentiation in cortico-striatal circuits (Calabresi et al., 1999). A time-limited insult to the nigrostriatal system can set in motion a self-perpetuating process of neuro-degeneration involving glial cells and NO (Hirsch and Hunot, 2000). Furthermore, the role of NO and cGMP in determining the long-term response to a psychotropic agent has been suggested (Harvey, 1996).

Haloperidol directly inhibits neuronal NO-synthase (nNOS) in vitro (Hu et al., 1994). Recent studies have also reported the ability of NOS inhibitors to induce catalepsy in
rodents and to potentiate or modify haloperidol-induced catalepsy (Del-Bel et al., 1998; Del-Bel and Guimarães, 2000). These studies strongly suggest that critical nNOS activating pathways in the brain are affected during chronic haloperidol treatment and that the NO-cGMP pathway is implicated in the neuropharmacologic mechanism mediating this response. Since haloperidol has been found to persist in brain tissue, this study has attempted to determine whether a 3 week withdrawal of the drug after 17 weeks of treatment can induce any enduring effects on striatal cGMP levels. To further characterize the role of cGMP in this response, early-stage (weeks 1-3) disruption of striatal cGMP will be induced using the NO-cGMP inhibitory agent, methylene blue (Moore and Handy, 1997; Volke et al., 1999), and assessing the resulting effects on locomotor behavior as well as on striatal cGMP. Furthermore, methylene blue is able to modify brain guanylyl cyclase activity in vivo (Masaki and Kondo, 1999) with significant behavioral sequelae (Eroglu and Caglayan, 1997). Using methylene blue may delineate a possible cGMP-dependent mechanism whereby chronic extrapyramidal symptoms may adversely predict long-term outcome insofar as severity of motor side effects is concerned. In order to relate any possible effects of early inhibition of NOS/cGMP over time on central and peripheral NO-cGMP activity, striatal cGMP levels and serum nitrogen oxides (NOx) were assayed as surrogate markers of NO-cGMP activity. Since haloperidol has been found to persist in brain tissue after discontinuation of the drug, this approach could elucidate whether late-onset haloperidol effects on behavior involve continual central effects of the drug on the NO pathway.

MATERIALS and METHODS

Reagents and drugs

Haloperidol was purchased from Sigma (St. Louis, MO, U.S.A.) and methylene blue 1% solution was purchased from Kyron Laboratories (Johannesburg, South Africa). The methylene blue was sterilized by filtration and stored in multi-dose vials prior to administration. An appropriate amount of haloperidol was first dissolved in glacial acetic acid and diluted with distilled water to volume and stored in opaque bottles. The pH was checked to be above pH 4.6. Haloperidol was prepared fresh every week. All other reagents were analytical grade. Cadmium granules were purchased from Riedel-de Haen, Seelze, Germany, while glycine and N-naphthyl ethylenediamine dihydrochloride (NEDA) were purchased from SAarchem, Krugersdorp, South Africa. Sulphanilamide was purchased from E. Merck, Darmstadt, Germany.

Animals

The study protocol was approved by the Ethics Committee for Research on Experimental Animals of the University of Potchefstroom. Male Sprague-Dawley rats, initially weighing between 200-250g, were housed one per cage at the Animal Research Center of the university. The animals were kept under constant conditions of temperature (21±5°C), humidity (50±10%) and light requirements (12hr light-dark cycle). They were allowed free access to food and water.
Drug treatment

Haloperidol was administered to the animals in their drinking water. The concentration of the haloperidol stock solution was adjusted to allow a daily haloperidol intake of 0.25mg/kg/d. Methylene blue was administered via intraperitoneal (ip) injection at a dose of 5mg/kg/d (Eroglu and Caglayan, 1997) for a maximum of three weeks (to limit undue injection stress). During chronic treatment, two groups of 12 rats each were allocated to 17 weeks chronic oral haloperidol exposure, while a third group of 12 rats received water only. In the haloperidol-treated groups, methylene blue (5mg/kg/d) or saline (ip), were co-administered with haloperidol for the first three weeks of haloperidol treatment. At week 17, haloperidol was withdrawn, whereupon the animals received water until week 20. All animals were included in both behavioral and biochemical analyses.

Behavioral measures

Haloperidol-induced locomotor behavior was determined as described previously (Harvey and Bester, 2000) by assessing the degree of oral vacuous chewing movements (VCM's). Mature rats exhibit low frequency jaw movements characterized by bursts of seemingly purposeless repetitive opening and closing of the jaws (Rosengarten et al., 1999). These oral movements can be exacerbated by chronic administration of typical neuroleptics (Rosengarten et al., 1999). This behavioral method has been effectively used as an animal equivalent of human extrapyramidal symptoms and tardive dyskinesia (Egan et al., 1995). Briefly, VCM rating sessions were undertaken between 08h00 and 12h00 after a habituation period of 4 min. Each animal was then placed into a raised transparent cage (34 x 18 x 24 cm) and assessed for 2 min by four raters placed diagonally opposite one another. VCM behavior was scored as the following: jaw tremors, chew-bursts (more than one chew action that rapidly follow one another), single chew movements, tongue protrusions, grooming and rearing. Individual scores were then totaled to represent total VCM's. Rearing and grooming were negligible and eventually excluded from the total VCM score. For the chronic treatment cohort, VCM’s were assessed weekly for the first 4 weeks, then every two weeks until week 14. Between weeks 14-17, VCM’s were assessed weekly and from week 17-20, it was performed every third day.

Extraction and assay of striatal cGMP

The extraction and assay of cGMP followed that described by Harvey et al., (1994). Briefly, animals were sacrificed by decapitation and the brains rapidly removed on an ice-cooled dissection slab. The striata were then dissected out and immediately frozen in liquid nitrogen and stored at -70°C. After sufficient samples had been collected, a batch cGMP assay was performed. Two fixed striatal tissue preparations were randomly pooled and homogenized for 1 min in 50mM TRIS/HCl buffer containing 4mM EDTA, pH 7.5 (100mg tissue/ml) using a Heidolph glass-tetlon homogenizer. The homogenate was then heated at 100°C for 3 min. These latter manipulations (Ca²⁺-chelation and heat) were essential to inactivating cGMP phosphodiesterases present in the extract (Harvey et al., 1994). The homogenates were then centrifuged at 2000g for 45 min in an SME refrigerated centrifuge. Thereafter, the resulting supernatant was used for the assay of cGMP using a commercially
available kit (Amersham, UK). Data are expressed as pmol cGMP/g wet weight of the striatal extract.

Collection of blood and determination of serum NOx

Serum was obtained from trunk blood collected at sacrifice, as described by Cortas and Wakid (1990). Blood was collected in EDTA (K3) vacutainers and, within 1 hr, centrifuged at 1000rpm for 10min. The separated serum was then stored at -18°C. Assay of serum NOx was performed using a modified Greiss procedure described earlier (Harvey and Bester, 2000), where serum NO3 - is reduced by cadmium to NO2 -; derivatised with sulphanilamine and quantified by spectrophotometric analysis after azo-coupling with NEDA. Absorbance of the resulting chromophore was determined at 545nm using a Gilford Stassar III Spectrophotometer. A plot of absorbance against NO3 - was linear with a regression coefficient of >0.98 and an inter-assay variation of <5%. Total sample NO3 - expressed as uM was determined by subtracting the measured concentration of NO2 - and multiplying by the appropriate dilution factor.

Statistical analyses

All VCM data and biochemical parameters were analyzed using a repeated measures analysis of variance (rmANOVA) followed by Tukey's Studentized Range Test (SAS, 1988). A 95% confidence interval (p<0.05) was used to define significance.

RESULTS

Biochemical studies

Chronic haloperidol plus saline (week 1-3), effect on cGMP and NOx levels: After the 3 week withdrawal period (week 20), striatal cGMP levels were significantly lower in the haloperidol-treated animals compared to saline control (p<0.05; Fig. 1). Moreover, haloperidol alone also significantly suppressed serum NOx levels compared to control animals (p<0.05; Fig. 2).

Chronic haloperidol plus methylene blue (week 1-3), effect on cGMP and NOx levels: Three weeks after withdrawal, striatal cGMP levels were significantly lower than that of the saline control group (p<0.05; Fig. 1), and also lower than that induced by haloperidol alone, although significance was not attained (p>0.05; Fig. 1). Plasma NOx values in the haloperidol plus methylene blue group, however, were unchanged compared to control groups (Fig. 2), but markedly higher than haloperidol alone (p<0.05; Fig. 2).

Behavioral studies

Chronic haloperidol plus saline (week 1-3), effect on locomotor behavior: Chronic haloperidol alone induced significantly higher VCM's than saline control, peaking within 1 week and maintaining a steady plateau yet remaining significantly higher than control (p<0.05; Fig. 3). Acute withdrawal of haloperidol at week 17 was associated with a steady decline of VCM's (Fig. 3).
**Figure 1.** Comparative striatal cGMP data for the different drug treatments, as indicated, during a 17 week treatment period, followed by 3 week withdrawal. Cyclic GMP is expressed as pmol/g wet weight (mean ± SEM). */** = Significant differences (p<0.05).

**Figure 2.** Comparative serum NOx data for the different drug treatments, as indicated, during a 17 week treatment period, followed by 3 week withdrawal. NOx are expressed as uM (mean ± SEM). */** = Significant differences (p<0.05).
Chronic haloperidol plus methylene blue (week 1-3), effect on locomotor behavior

A two-factorial test (time-dose; time-treatment) with rmANOVA revealed the superiority of haloperidol plus early administration of methylene blue compared to saline control, in causing a significantly greater number of VCM's throughout the 20 week period (p<0.05; Fig. 3). The co-administration of methylene blue with haloperidol for the first 3 weeks of the treatment protocol also resulted in significantly higher VCM scores than haloperidol alone and which were maintained throughout the 17 week treatment period, even after methylene blue discontinuation at week 3 (two-factorial rmANOVA; p<0.05, Fig. 3). Discontinuation of haloperidol at week 17 resulted in a rapid diminution of VCM's.

![Graph](image)

**Figure 3.** Comparative VCM data for the different drug treatments, as indicated, during a 17 week treatment period, followed by 3 weeks withdrawal. VCM's are expressed as the total number of VCM's/2min (mean ± SEM). Refer to "Results" for statistical data.

**DISCUSSION**

This study has investigated a putative role for NO-cGMP in the induction of haloperidol-induced extrapyramidal symptoms in a rat behavioral model. Since both humans and animals exposed to long-term anti-psychotics display a risk of developing tardive dyskinesia.
subsequent to extrapyramidal symptoms (Egan et al., 1995; Harvey et al., 1999), the aim of the present study was to evaluate the role of NO and/or cGMP as possible early biochemical determinants in this response. Haloperidol has been found to accumulate in brain long after discontinuation of treatment. The current study attempts to bring together a biochemical association with this event that may explain the worsening of extrapyramidal symptoms in many patients subsequent to discontinuing the drug.

Haloperidol is a potent inhibitor of nNOS in vitro (Hu et al., 1994; Moore and Handy, 1997), while specific nNOS inhibitors have been found to exacerbate haloperidol-induced catalepsy (Del-Bel et al., 1998; Del Bel and Guimaraes, 2000). The NO-pathway, therefore, appears to occupy a central role in either maintaining normal movement or preventing the biochemical precursors that initiate neuronal damage and eventual irreversible motor dysfunction. In the present study, long-term haloperidol administration for 17 weeks resulted in significant VCM’s. An essential feature of human tardive dyskinesia, and one which we attempted to simulate in the present study, is that it can be brought about or exacerbated by sudden withdrawal of the antipsychotic (Gibbon and Swanepoel, 1997). In animals, exacerbation is usually evident after an extended withdrawal period of 24-30 weeks (Egan et al., 1995). Possibly due to the short withdrawal period, VCM’s were not increased between weeks 17-20. However, striatal cGMP levels, as well as serum NO, levels, were significantly reduced. This data not only lends support for haloperidol’s ability to accumulate in brain tissue long after discontinuation (Cohen et al., 1992; Kornhuber et al., 1999), but to also induce protracted pharmacological effects post-withdrawal. Moreover, it also suggests that acute withdrawal is associated with biochemical changes in the striatum that may underlie the later development of worsening motor dysfunction. Of significance in this study was that exposure of the animals to methylene blue in the first three weeks of haloperidol treatment was associated with significantly greater motor abnormalities (total VCM) than haloperidol alone over the total treatment period. This response implies a strong association with cGMP suppression. In a previous study, sub-acute administration of methylene blue for 3 weeks, while not associated with the induction of VCM’s on its own, succeeded in amplifying the VCM response to 3 weeks haloperidol exposure. Furthermore, this behavioral response was also associated with a marked suppression of striatal cGMP compared to both haloperidol-treated and control animals (Harvey and Bester, 2000). In the present study, striatal cGMP levels were slightly lower than that of haloperidol alone, although significance was not reached, but still significantly lower than control values. We conclude that an early sub-acute disruption of cGMP transduction, brought about by methylene blue, may have an etiological association with aberrant motor function associated with chronic haloperidol. The early-onset perturbation of the NO-cGMP pathway using methylene blue and the resulting exacerbation of VCM’s over the whole treatment period also imply that haloperidol, and the haloperidol - methylene blue combination, modifies some form of long-term motor learning involving cGMP.

Of interest is that, although haloperidol alone induced a suppression of both striatal cGMP and serum NOx, methylene blue challenge to haloperidol-treated animals appeared to maintain suppression of striatal cGMP, although the combination did not induce a similar suppressive effect on peripheral serum NOx. Unlike the central effects of haloperidol, a
peripheral effect appears to be lost over the ensuing 4 months treatment. However, this combination also appeared to prevent the haloperidol-induced suppression of NOx as seen in the haloperidol group alone. Haloperidol has been found to exert a biphasic effect on nNOS, inhibiting enzyme activity at lower concentrations, while higher concentrations are stimulatory (Borda et al., 1999). This observation may reflect inhibition-induced up-regulation of the Ca\(^{2+}\)/calmodulin-dependent NO-guanylyl cyclase system (Hu et al., 1994).

In this case, the higher NOx levels described for the haloperidol - methylene blue group compared to haloperidol alone may be due to a reactive increase in NOS activity due to an initial overt suppression of the enzyme by combined haloperidol plus methylene blue exposure. In addition to the well-recognized enzymatic synthesis of NO via NOS, recent evidence suggests that NO can also be synthesized via a non-enzymatic route in the periphery (Nagase et al., 1997). Thus, since the enzymatic pathway is directly affected by the NOS inhibitory action of haloperidol and methylene blue, in an attempt to maintain vascular homeostasis, the non-enzymatic pathway may over-compensate for the severely diminished activity of the former resulting in a raised NOx values. In support of this, Suto et al., (1995) report that urinary NOx values actually rise when the NOS inhibitor, L-nitroarginine methyl ester, is administered to rats.

The fact that significant effects on striatal cGMP and locomotor behavior were maintained or exacerbated with the combination of early methylene blue administration plus haloperidol, emphasizes that methylene blue -inhibition of the nNOS-cGMP pathway augments a similar form of nitricergic hypofunction induced by chronic haloperidol. Moreover, these effects were restricted to the central nervous system, thereby implicating the induction by haloperidol alone, but especially the haloperidol - methylene blue combination, of a long-lasting form of neuronal plasticity mediated by the loss of NO and/or cGMP from neuronal systems within the striatum. One attenuated mechanism as a result of such inhibition has particular relevance, viz. cGMP-mediated release of striatal DA (Guevara-Guzman et al., 1994). This response may be important in preventing the eventual neurological sequelae of chronic D\(_2\) receptor blockade by haloperidol and other antipsychotics. Cyclic GMP is also directly involved in long-term potentiation (Arancio et al., 1995; Calabresi et al., 1999), which may be responsible for the behavioral effects of early methylene blue administration to chronic haloperidol treatment. However, alternative pathways in tardive dyskinesia development may also involve direct NO-mediated events. By down-regulating the NO-cGMP pathway, chronic haloperidol allows conditions that will capitalize on the absence of the anti-oxidative properties of NO (reviewed by Gorden, 1998) resulting in the perpetuation of neuronal oxidative stress processes and the induction of tardive dyskinesia (Harvey and Bester, 2000). Alternatively, nNOS inhibition may increase the release of NO from inducible NOS resulting in cytotoxic amounts of NO being released (Colasanti and Suzuki, 2000).

In conclusion, chronic haloperidol-associated motor changes are associated with profound suppressive effects on striatal cGMP that persists post-withdrawal, and which can be exacerbated by early addition of a nNOS/guanylate cyclase inhibitor. Furthermore, this cGMP suppression represents an event that takes place early in treatment, yet which exerts its effects throughout the treatment period in the form of an exacerbation of extrapyramidal
effects. Since tardive dyskinesia is linked in a time-dependent manner to chronic extrapyramidal symptoms, these data may have relevance to the neurobiology of antipsychotic-induced extrapyramidal symptoms and the later risk of inducing tardive dyskinesia.

ACKNOWLEDGMENTS

The authors would like to thank the South African Medical Research Council for financial assistance, as well as Cor Bester, Antoinette Fick and Dr. Douw van der Nest for assistance in the setting up and determination of all behavioral measures.

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