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Haloperidol- and clozapine-induced oxidative stress in the rat brain

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Abstract

Haloperidol (HAL) is a typical neuroleptic that acts primarily as a D_2 dopamine receptor antagonist. It has been proposed that reactive oxygen species play a causative role in neurotoxic effects induced by HAL. Adult male Wistar rats received daily injections of HAL (1.5 mg/kg) or clozapine (CLO, 25 mg/kg), an atypical neuroleptic, for 28 days. Control animals were given saline (SAL; NaCl 0.9%). Oxidative parameters in the brain were measured in the striatum (ST), hippocampus (HP) and cortex (CX). Thiobarbituric acid (TBA) reactive substances (TBAR) levels were increased by HAL treatment in the ST and decreased in CX of both of the HAL- and CLO-treated rats. Protein carbonyls were significantly increased by both HAL and CLO in the HP. The nonenzymatic antioxidant potential was decreased in the HP, and superoxide production was significantly increased in the ST following treatment with HAL. CLO induced an increase in superoxide production in the HP. Neither HAL nor CLO affected catalase (CAT) and superoxide dismutase (SOD) activities. The findings suggest that HAL and CLO can induce oxidative damage to the ST and HP in rats.
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Keywords: Haloperidol; Clozapine; Oxidative stress; Reactive oxygen species

1. Introduction

Haloperidol (HAL) is often prescribed for the treatment of the positive symptoms of schizophrenia. HAL is a butyrophenone that acts primarily as a D_2 dopamine receptor antagonist. Like most typical neuroleptics, HAL can cause extrapyramidal symptoms, including tardive dyskinesia (TD). The development of TD can be attributed to the potential toxic effects of prolonged typical neuroleptic administration. It has been shown that high concentration of this dopamine D_2 receptor antagonist is cytotoxic for various cell types (Vilner et al., 1995). This could occur via an oxidative stress mechanism following the production of inhibitors of mitochondrial respiration (Rollema et al., 1994; Yokoyama et al., 1998). It is known that haloperidol pyridinium ion derived metabolite (HP^+) severely affects the transport of dopamine by blocking the dopamine receptors (Creese et al., 1976).

It has been shown that HP^+ is a potent inhibitor of complex I and can interfere with electron transport at both complexes I and II (Rollema et al., 1994). Furthermore, it has been suggested that the increase of dopamine turnover, which, in turn, could result in increased production of toxic dopamine metabolites, may contribute to HAL neurotoxicity (Westerink and Vries, 1989; Yokoyama et al., 1998). Because the enzymatic dopamine metabolism generates hydrogen peroxide, increased dopamine turnover could lead to overproduction of hydrogen peroxide. Hydroxyl radical,

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which can be produced from hydrogen peroxide by the catalytic action of transition metals such as iron, is a strong neurotoxin, even in a small amount (Halliwell, 1992).

Clozapine (CLO) is an atypical (or "novel") neuroleptic drug with serotonin/dopamine antagonistic properties associated with substantially fewer extrapyramidal symptoms than conventional antipsychotics do (Tuunainen et al., 2002).

In addition to inducing cytotoxic and motor effects, it has been shown recently that both typical and atypical neuroleptics can alter cognitive function in both human and animal models. For instance, HAL and olanzapine impair spatial memory in a water maze (Skarsfeldt, 1996; Terry et al., 2002), and HAL, CLO and risperidone impair the acquisition of a radial-arm maze in rats (Rosengarten and Quatermain, 2002), although a beneficial effect of risperidone on memory when compared with HAL has been shown (Nowakowska et al., 1999). In humans, recent evidence suggests that schizophrenic patients treated with atypical antipsychotics may perform better in cognitive tasks when compared with patients treated with typical antipsychotics (Beuzen et al., 1999; Velligan et al., 2002).

The aim of the present study was to evaluate effects of a 28-day treatment with a typical (HAL) and an atypical (CLO) neuroleptic administration on the levels of lipid peroxidation [thiobarbituric acid reactive substances (TBARS)], protein carbonylation, catalase (CAT) and superoxide dismutase (SOD) activities, mitochondrial superoxide generation and total radical trapping antioxidant parameter (TRAP) in the hippocampus (HP), cortex (CX) and striatum (ST).

2. Material and methods

2.1. Animals

Thirty male Wistar rats (2-month old) were obtained from the State Foundation for Health Science Research (FEPPS/LACEN-RS, Porto Alegre, Brazil). Animals were housed five to a cage (size of home cages: $50 \times 35 \times 16$ cm), with food and water available ad libitum, and were maintained on a 12-h light/dark cycle (lights on at 07:00 h). All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care, with the approval of the local ethics committee.

2.2. Drugs and chemicals

LC-grade water obtained in Milli-Q system, Millipore, Bedford, MA, USA. Thiobarbituric acid (TBA), CAT, SOD, dinitrophenylhidrazine (DNPH), adrenaline, hydrogen peroxide, luminol (3-aminophthalhydrazide) and succinate were purchased from Sigma, St. Louis, MO. 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Aldrich Chemical, Milwaukee, WI. Glycine was purchased from Nuclear, Diadema, SP, Brasil. HAL (Haldol) was purchased from Critalina. CLO (Leponex) was purchased from Novartis Biosciences.

2.3. Pharmacological procedures

The animals were divided in three experimental groups: control, HAL and CLO groups ($n = 10$ animals per group). Animals received daily intraperitoneal injections of HAL (1.5 mg/kg) or CLO (25 mg/kg) in a 1.0 ml/kg volume for 28 days. Control animals received saline (SAL; NaCl 0.9%, 1.0 ml/kg). Three days after the last injections, the rats were sacrificed by decapitation, and the HP, CX and ST were immediately removed. The parameters of free-radical damage to biomolecules (TBARS and protein carbonylation), TRAP, mitochondrial superoxide production, SOD and CAT activities were then measured in those brain structures.

2.4. Thiobarbituric acid reactive species (TBARS)

As an index of ROS production, we used the formation of TBARS during an acid-heating reaction, which is widely adopted as a sensitive method for the measurement of lipid peroxidation, as previously described (Draper and Hadley, 1990). Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% (TCA) and 1 ml of TBA 0.67%, then heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm. Results are expressed as malondialdehyde (MDA) equivalents (nmol/mg protein).

2.5. Measurement of protein carbonyls

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with DNPH, as previously described (Levine et al., 1990). Briefly, proteins were precipitated by the addition of 20% TCA and redissolved in DNPH and the absorbance read at 370 nm.

2.6. Measurement of mitochondrial superoxide generation

Submitochondrial particles (SMP) were isolated from the brain structures studied by differential centrifugation, as previously described (Poderoso et al., 1996). Superoxide was estimated by measuring adrenaline oxidation in a buffer containing SMP, succinate (as electron transfer chain initiator) and CAT. To assure assay specificity, a negative control was made in the presence of SOD.

2.7. Total reactive antioxidant potential (TRAP)

The antioxidant potential of the brain structures was estimated by the total radical-trapping antioxidant parameter (TRAP). The principle of TRAP measurement has been previously described (Wayner et al., 1985). Briefly, the reaction was initiated by injecting luminol and AAPH—a free-radical source that produces peroxyl radical at a con-

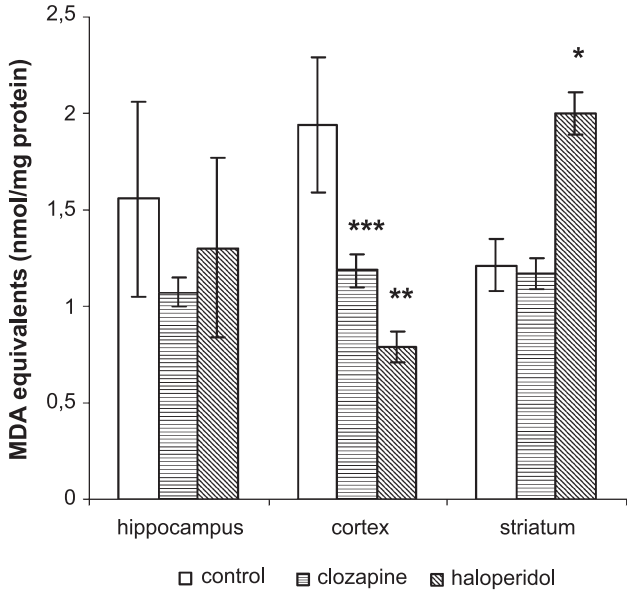

Fig. 1. TBARS in the hippocampus (HP), cortex (CX) and striatum (ST) of rats following HAL and CLO treatment. Animals received daily intraperitoneal injections of 1.5 or 25 mg/kg of HAL or CLO for 28 days. Control animals received SAL. Animals were sacrificed by decapitation, and the HP, CX and ST were immediately removed for the determination of TBARS as described under Material and methods. Values are expressed as means ± S.E.M. ($n = 10$). * Significant difference from the SAL group ($P < .05$); * * significant difference from both SAL and CLO ($P < .05$); * * * Significant difference from both SAL and HAL ($P < .05$).

stant rate—in glycine buffer, which resulted in steady luminescence emission. The addition of the brain structure homogenates decreases the luminescence proportionally to its antioxidant potential. The protein content of the brain structure homogenates was determined (Lowry et al., 1951). The luminescence emission was followed for 60 min after the addition of the brain structure homogenates (150 μg of protein). Chemiluminescence was read in a liquid scintillation counter (Wallace 1409) as counts per minutes (cpm).

2.8. Antioxidant enzyme activities

For SOD activity, brain structures were homogenized in 50 mM glycine buffer (pH 10.2). SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (Bannister and Calaberese, 1987).

To determine CAT activity, brain structures were sonicated in 50 mM phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at $3000 \times g$ for 10 min. The supernatant was used for enzyme assay. CAT activity was assayed by measuring the rate of decrease of H_2O_2 absorbance at 240 nm (Dal-Pizzol et al., 2001a,b). All results were normalized by the protein content (Lowry et al., 1951).

2.9. Statistical analysis

All data are presented as means ± S.E.M. Differences among experimental groups in experiments evaluating oxidative parameters were determined by one-way ANOVA, multiple comparisons were performed by a Newman–Keuls test. In all experiments, P values less than .05 were considered to indicate statistical significance.

3. Results

3.1. Oxidative damage parameters

Fig. 1 shows an increase in TBARS in the striatum after HAL treatment. However, there was a decrease of TBARS levels induced by both HAL and CLO treatments in the cortex. TBARS levels were also determined in the SMP but there were no significant differences among groups (data not shown). In addition, both HAL and CLO induced an increase in protein carbonyls in the HP compared with the control group (Fig. 2). These data suggest that HAL and CLO can induce differential effects on oxidative stress parameters in different CNS structures, which could be related to the toxic properties of those antipsychotics.

3.2. Measurement of mitochondrial superoxide generation

Because HAL or its metabolites could inhibit complex I and affect electron transport at both complexes I and II, we determined mitochondrial superoxide generation as an index

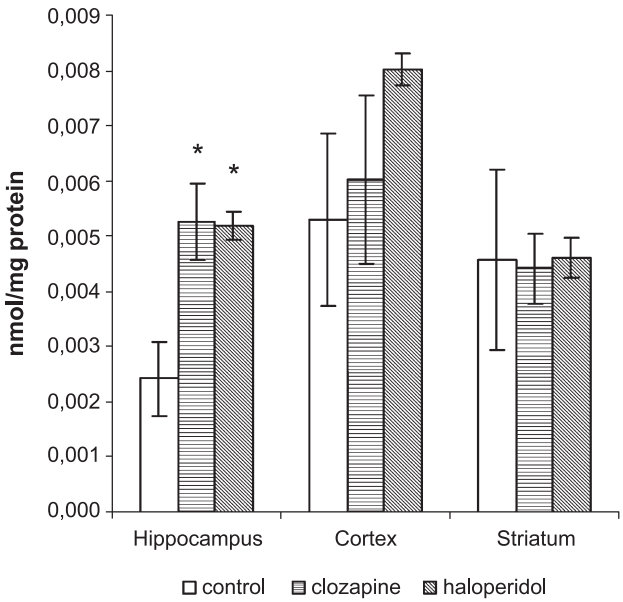

Fig. 2. Protein carbonyls in hippocampus (HP), cortex (CX) and striatum (ST) of rats following HAL and CLO treatment. Animals received daily intraperitoneal injections of 1.5 or 25 mg/kg of HAL or CLO for 28 days. Control animals received SAL. Animals were sacrificed by decapitation, and the HP, CX and ST were immediately removed for the determination of protein carbonyls as described under Material and methods. Values are expressed as means ± S.E.M. ($n = 10$ each group). * Significant difference from the SAL group ($P < .05$).

of uncoupling of electron transfer chain. We observed an increase in mitochondrial superoxide production in the ST following HAL treatment (Fig. 3), supporting the possibility that HAL induced oxidative stress in specific CNS areas. CLO administration induced an increase in mitochondrial superoxide generation in the HP; however, this effect was probably not associated with biomolecule damage (see results on TBARS and protein carbonyls) or mitochondrial oxidative stress (as demonstrated by mitochondrial TBARS).

3.3. Total reactive antioxidant potential (TRAP)

There was a decrease in the HP total antioxidant potential induced by HAL on the first 10 min of the TRAP assay (Fig. 4A). This shows a decrease on the nonenzymatic antioxidant defenses on the HP in response to HAL administration. Probably, this reflects an adaptation to HAL-induced oxidative stress. TRAP values were not different between treatment in the CX or ST (Fig. 4B and C).

3.4. Antioxidant enzyme activities

Antioxidant enzyme activities CAT and SOD were affected by neither HAL nor CLO in all CNS portions studied (data not shown). This inability to increase enzymatic antioxidant defenses in response to oxidative damage could, in part, explain the effects of HAL on oxidative parameters in the HP and ST.

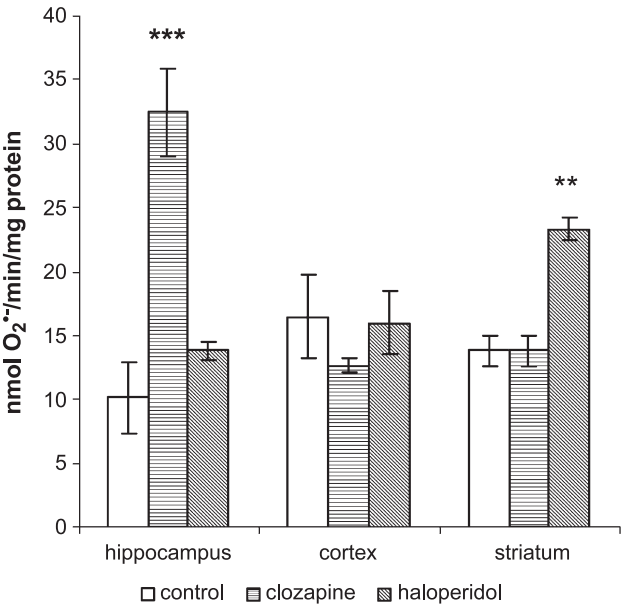

Fig. 3. Mitochondrial superoxide generation in the hippocampus (HP), cortex (CX) and striatum (ST) of rats following HAL and CLO treatment. Animals received daily intraperitoneal injections of 1.5 or 25 mg/kg of HAL or CLO for 28 days. Control animals received SAL. Animals were sacrificed by decapitation, and the HP, CX and ST were immediately removed the for determination of mitochondrial superoxide generation, as described under Material and methods. Values are expressed as means ± S.E.M. ($n = 10$ each group). * * significant difference from both SAL and CLO ($P < .05$); * * * significant difference from both SAL and HAL ($P < .05$).

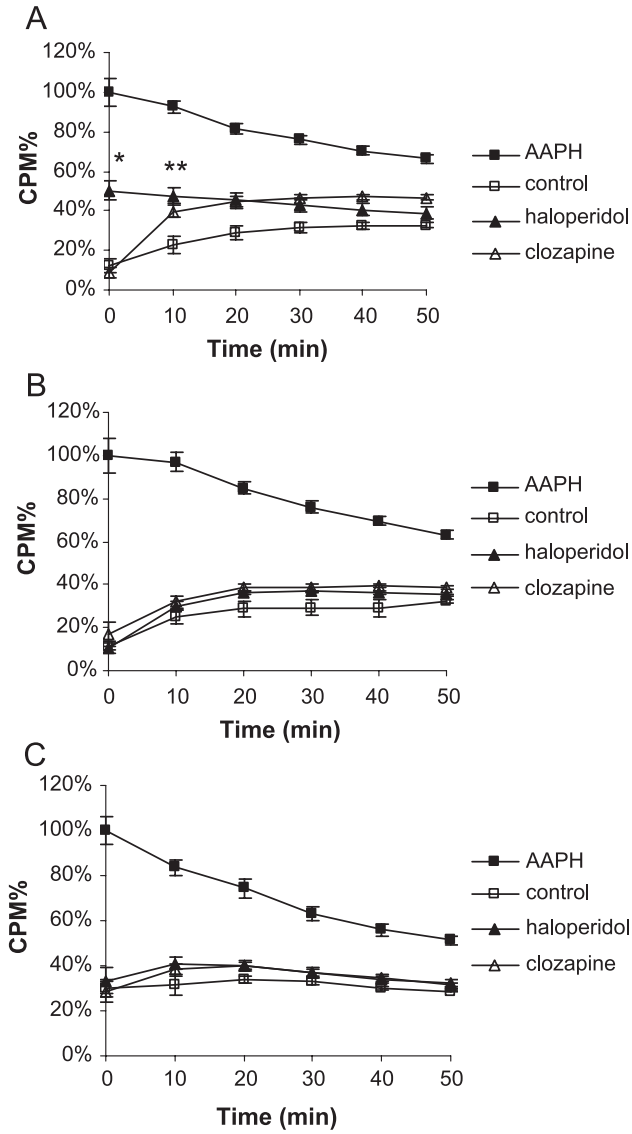

Fig. 4. TRAP in the hippocampus (HP), cortex (CX) and striatum (ST) of rats following HAL and CLO treatment. Animals received daily intraperitoneal injections of 1.5 or 25 mg/kg of HAL or CLO for 28 days. Control animals received SAL. Animals were sacrifices by decapitation, and the HP (A), CX (B) and ST (C) were immediately removed. Brain structure hemogenates (150 μg of protein) were added to a solution AAPH and luminol, and the chemiluminescence was followed by 60 min. The decrease in the chemioluminescence is proportional to the antioxidant potential. Values are expressed as means ± S.E.M. Values are expressed as percentage of control (AAPH) = 100% ($n = 5$). * Significant difference from the SAL group ($P < .05$); * * significant difference from both SAL and CLO ($P < .05$).

4. Discussion

On the basis of previous findings, a free-radical-dependent mechanism was postulated suggesting that HAL-induced

neurotoxicity may be mediated by blockade of mitochondrial respiration respiratory chain, particularly in complexes I and II (Burkhardt et al., 1993; Arnaiz et al., 1999). Rollema et al. (1994) provided evidence that a key metabolite, haloperidol pyridinium (HP^+), shared some structural similarity and toxic actions with MPP^+ (1-methyl-4-phenyl-1,2-dihydropyridinium ion), a toxic metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine), which is widely used to induce experimental neurodegeneration in rodents and primates. It is possible that HP^+, like MPP^+, is a potent inhibitor of complex I and can interfere with electron transport at both complexes I and II (Rollema et al., 1994).

Our results show that HAL treatment is followed by an enhancement of $O_2^{\cdot -}$ formation by mitochondria in the rat ST. The uncoupling of oxidative phosphorylation and the increase in superoxide production rate are probably related to the multiple effects of toxins upon mitochondrial electron transfer and ATP synthesis (Ritter et al., 2003). One of these effects should be an inhibition of the electron transfer at the ubiquinone-cytochrome b region, which is required to elicit a significant production of superoxide (Poderoso et al., 1996). It has been reported that HAL treatment can lead to enhance nitric oxide (NO) production (Krzascik and Kostowski, 1997). NO could reversibly bind to ubiquinone-cytochrome b uncoupling electron transfer, leading to superoxide production (Poderoso et al., 1996). Superoxide could react with NO to form peroxynitrite, which, in turn, binds irreversibly to ubiquinone-cytochrome b, initiating a cascade of mitochondrial superoxide production (Ritter et al., 2003). Increased superoxide production in the mitochondrial membrane has been indicated as a pathogenic cause for several CNS disorders, including Parkinson and TD (Tritschler et al., 1994). These findings contribute with the hypotheses that HAL increases free-radical production, leading to oxidative stress.

The inherent biochemical and physiological characteristics of the brain, with high unsaturated phospholipid content and energy requirement, make it particularly susceptible to free-radical-mediated damage (Arnaiz et al., 1999). We showed here that HAL treatment caused an increase in lipid peroxidation levels in the ST. This increase is probably due to the enhanced $O_2^{\cdot -}$ production also found in the ST. Lipid peroxidation has been implicated in the toxic effect of many chemicals and in many tissue injuries and disease processes (Dal-Pizzol et al., 2001a,b). It has been suggested that reactive oxygen species could be involved in neuronal damage by inducing an increase in lipid peroxidation. Moreover, lipid peroxidation could be responsible for the loss on the membrane permeability (Dal-Pizzol et al., 2000).

Although HAL altered TBARS levels in the ST, our results do not show changes in TBARS levels in the SMP, suggesting that HAL does not affect mitochondria integrity. An explanation for this discrepancy is that HAL-induced oxidative damage in lipids affects mostly unsaturated phospholipids, which are present in high concentration on the neuronal membrane (Arnaiz et al., 1999). Both HAL and CLO induced a decrease in TBARS levels in the CX. This finding might be related to a reduction in cortical activity induced by antagonism of dopamine receptors (Desco et al., 2003).

Intriguingly, both HAL and CLO induced protein carbonylation in the HP. It is the first demonstration of oxidative damage on proteins induced by HAL and CLO. This result could, in part, explain the cognitive deficits in memory tasks sensitive to the damage to the HP in rats treated with antipsychotics. Further studies should clarify the mechanisms underlying the effects of CLO and HAL on hippocampal function.

It is well known that the brain is relatively poorly endowed with protective antioxidant enzymes or antioxidant compounds (Halliwell and Gutteridge, 1989; Phillis, 1994). A low availability of antioxidant compounds against reactive oxygen species may explain why a higher antioxidant potential in TRAP assay was not observed. In addition, CAT and SOD activities were affected by neither HAL nor CLO. It has been reported that HAL treatment leads to an increase in H_2O_2 production by monoamine oxidase (MAO). Previous studies have shown that high concentrations of H_2O_2 inhibit SOD activity in a negative feedback mechanism. Furthermore, HAL treatment can lead to an increase in NO production (Krzascik and Kostowski, 1997), as mentioned before. A recent study showed that NO causes release of zinc from presynaptic boutons (Frederickson et al., 2002) and causes Zn^{2+} to be mobilized from zinc-binding proteins. In this way, the increase in NO production in the brain may lead to Zn^{2+} mobilization from SOD Cu/Zn, which could explain why SOD is not up-regulated, although superoxide production is enhanced. CAT is not usually regulated in the brain, its activity hardly ever changes in this tissue (Halliwell, 1992), and CAT is inhibited by superoxide (Kono and Fridovich, 1982), which could explain why CAT is not up-regulated in HAL-treated animals.

We were not able to find an extensive effect of CLO in the oxidative parameters we studied. The lack of influence of an atypical neuroleptic in the oxidative stress in the ST reinforces the hypotheses that HAL increases reactive oxygen species. The drug toxicity is not only due to its influence in dopamine receptor because CLO has the same property. For an unknown reason, we, for the first time, found enhanced superoxide production induced by CLO in the HP. Because CLO has affinity to other receptors in the CNS, such as $5\text{-}HT_{2A}$, the drug could induce superoxide generation in the HP through a mechanism that remains to be clarified.

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