Inactivation of Aconitase by Tetrahydrobiopterin in DArgic Cells: Relevance to PD

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ABSTRACT

Oxidative damage is thought to be a major cause of the progression of dopamine (DA)rgic neurodegeneration as in Parkinson's disease. We have previously reported that tetrahydrobiopterin (BH4), an endogenous molecule required for DA synthesis, exerts oxidative stress to DA-producing cells and facilitates the production of DA quinone. It is known that aconitase, present in both mitochondrial and cytosolic forms, act as an reactive oxygen species (ROS) sensor, and that their inactivation leads to further generation of ROS. In the present study we investigated whether the BH4-associated vulnerability of DA cells might involve aconitase. In DArgic cell line CATH.a, BH4 treatment caused reduction of activity of both mitochondrial and cytosolic aconitases, and this appeared to be due to direct inactivation of the pre-existing enzyme molecules. Although most of the activity reduced by BH4 was increased upon re-activation reaction under a reducing condition, the restoration was not complete, suggesting that irreversible and covalent modification has occurred. The aconitase inactivation was exacerbated in the presence of DA and attenuated in the presence of tyrosine hydroxylase inhibitor a-methyl-p-tyrosine, suggesting the involvement of DA. The degree of inactivation increased when the cells were treated with the quinone reductase inhibitor dicoumarol and decreased in the presence of quinone reductase inducer sulforaphane. Taken together, BH4 appeared to lead to both reversible and irreversible inactivation of aconitase and that this is facilitated by the presence of DA and accumulation of DA quinone.

Key words: aconitase, tetrahydrobiopterin, dopamine quinone, oxidative stress

INTRODUCTION

The exact cause of selective degeneration of dopamine (DA) cells in Parkinson's disease (PD) remains unknown, but free radical-mediated oxi-

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dative stress is thought to play a major role. Overwhelming evidence, particularly in post-mortem studies of human PD brain, indicates that oxidative damage evoked by reactive oxygen species (ROS) participates in the progression of DArgic neurodegeneration.

We have previously demonstrated that tetrahydrobioperin (BH4), an endogenous molecule required for DA synthesis, exerts oxidative stress to DA-producing cells (Choi et al., 2000; 2003; Lee et al.,

2007). BH4 undergoes oxidation during the hydroxylation reaction (Davis and Kaufman, 1993) as well as nonenzymatic auto-oxidation (Fisher and Kaufman, l973; Davis et al., l988) to produce hydrogen peroxide and superoxide radical. BH4 thus facilitates the oxidation of DA, producing DA quinone. Unless the quinone is removed by the enzyme quinone reductase, oxygen radicals are further formed during its redox cycling. As such, DA quinone is thought to cause mitochondrial dysfunction including disruption of membrane potential (Lee et al., 2002) and increased mitochondrial swelling (Berman and Hastings, 1999). In addition, DA quinone is thought to mediate α-synuclein-associated neurotoxicity in PD by covalently modifying α-synuclein monomer (Dunnett and Bjorklund, 1999) and by stabilizing the toxic protofibrillar α-synuclein (Conway et al., 2001).

The enzyme aconitase, an enzyme in the Krebs cycle, is known to be a sensitive index of oxidative stress. As a sulfur/iron protein, the enzyme is reversibly inactivated, resulting in slowing down of the Kreb's cycle. In addition, studies have demonstrated that the inactivation of mitochondrial aconitase results in generation of hydrogen peroxide and free iron (Flint et al.,1993; Vasquez-Vivar et al., 2000; Cantu et al., 2009). The free iron can mediate Fenton reaction, which in turn catalyzes further generation of intracellular ROS.

Based on this background information, it was possible to hypothesize that BH4 may lead to inactivation of mitochondrial aconitase and that this further contributes to the generation of ROS and cell death. We show in the present study that exposure to BH4 leads to both reversible and irreversible inactivation of aconitase and that this is facilitated by the presence of DA and accumulation of DA quinone.

MATERIALS AND METHODS

Materials

All culture media, fetal bovine serum (FBS), horse serum, L-glutamine, trypsin/EDTA, and penicillin-streptomycin were from GibcoBRL (Gaithersburg, MD, USA). BH4, sulforaphane, dicoumarol, isocitrate, α-methyl-p-tyrosine and ferrous ammonium sulfate were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals were reagent grade and were from Sigma or Merck (Rahway, NJ, USA).

Cell culture

CATH.a cells were grown in RPMI 1640 supplemented with 8% horse serum and 4% FBS. Cells were grown as monolayers in the presence of 100 IU/l penicillin and 10 μg/ml streptomycin at 37°C in 5% CO_2 in humidified atmosphere. For experiments, the cells were plated on polystyrene tissue culture dishes at a density of $1.5 \sim 2 \times 10^5$ cells/well in 24 well culture plates or 3×10^6 cells/60 mm plate. After 24 h, the cells were fed with fresh medium and treated with BH4 and/or other drugs.

Aconitase enzyme assay

Cells were lysed in ice-cold lysis buffer containing 0.6 mM $MnCl_2$, 1 mM L-cysteine, 1 mM citrate, and 0.5% Triton-X 100 in 50 mM Tris-HCl (pH 7.4). The aconitase activity was measured spectrophotometrically by monitoring the formation of cis-aconitate from isocitrate at 240 nm in 50 mM Tris-HCl (pH 7.4) containing 0.6 mM $MnCl_2$ and 20 mM isocitrate at 25°C (Gardner and Fridovich, 1992). Reactivation of aconitase was performed by incubating the cell lysate for 30 min in the presence of 50 mM dithiothreitol, 200 μM Na_2S, and 200 μM ferrous ammonium sulfate in 50 mM Tris-HCl (pH 8.0) at 37°C.

Mitochondrial fractionation

Mitochondrial fraction was prepared as described by Menzies et al. (2002). Cells grown on 100 mm culture dish were harvested and washed in phosphate buffered saline (PBS), homogenized on ice in 10 volume of 250 mM sucrose with 0.1 mM EGTA and 2 mM HEPES, pH 7.4, using a glass-Teflon homogenizer, and the homogenates were centrifuged at 900×g for 6 min at 4°C. Mitochondrial pellet and cytosolic fraction were obtained by centrifugation of the supernatant at 16,000×g for 10 min.

Data analyses

Comparisons were made using ANOVA and Newman-Keuls multiple comparisons test. $p < 0.05$ was considered statistically significant for all analyses.

RESULTS

BH4 leads to inactivation of aconitase

We first tested whether exposure of DArgic cells to BH4 might cause reduction of aconitase activity. CATH.a cells, the cell line extensively used to investigate DArgic cell death as an in vitro PD model, was treated with 100 μM BH4, as this concentration was previously determined to exert preferential toxicity to DArgic cells (Choi et al., 2000; 2003). As shown in Fig. 1A, the exposure to BH4

indeed resulted in a decrease in cellular aconitase activity: A reduction to 81% was obtained within 3 h, which was further decreased to 24 h. The reduction was already evident at a concentration as low as 10 μM (Fig. 1B). Since the exposure of cells to BH4 might have also caused a change in the amount of aconitase protein as well as inactivation of the pre-existing aconitase, we sought to examine the direct effect of BH4 on aconitase by subjecting lysate of untreated CATH.a cells to BH4. The results showed that this exerted a more immediate and
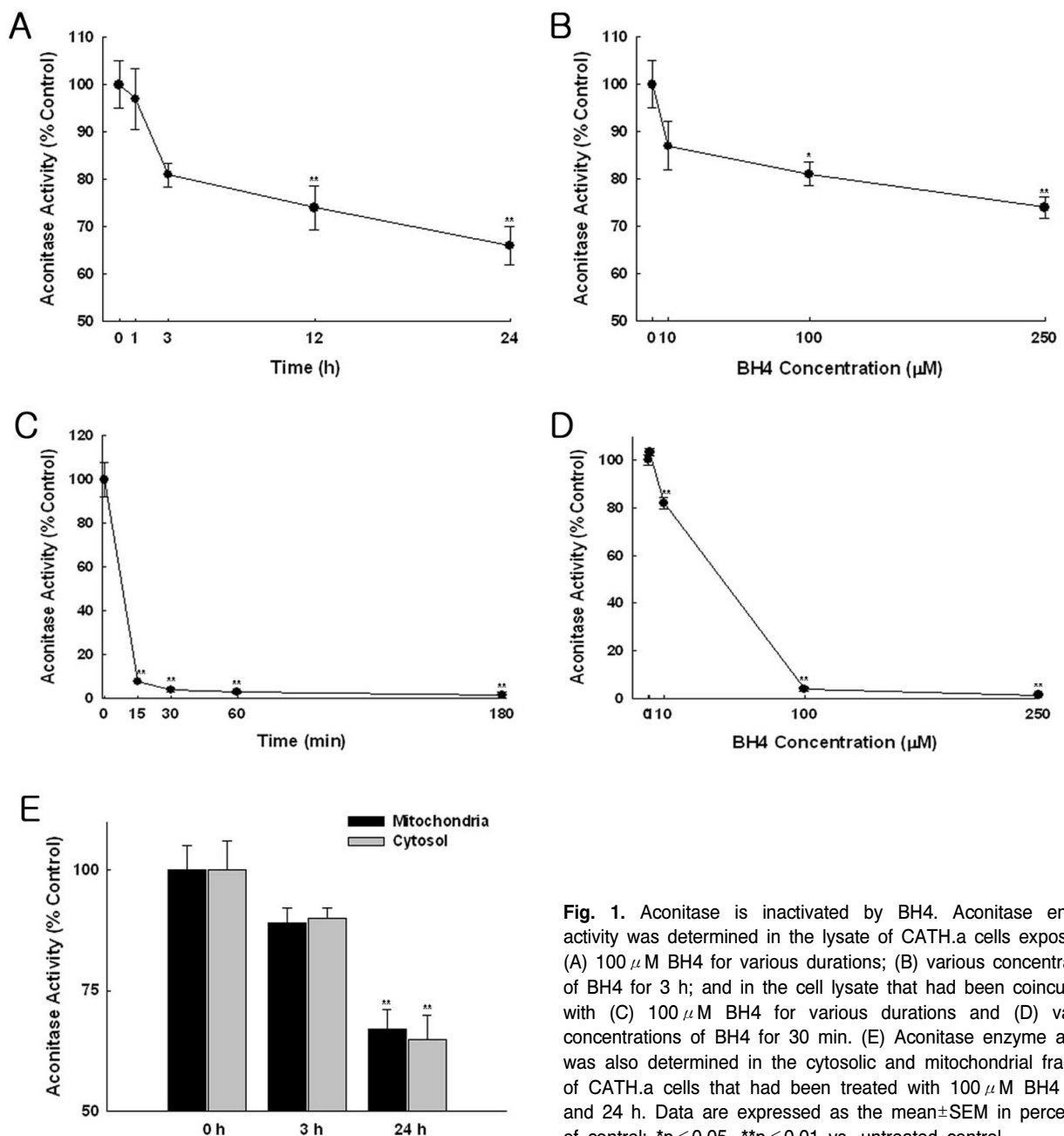

Fig. 1. Aconitase is inactivated by BH4. Aconitase enzyme activity was determined in the lysate of CATH.a cells exposed to (A) 100 μM BH4 for various durations; (B) various concentrations of BH4 for 3 h; and in the cell lysate that had been coincubated with (C) 100 μM BH4 for various durations and (D) various concentrations of BH4 for 30 min. (E) Aconitase enzyme activity was also determined in the cytosolic and mitochondrial fractions of CATH.a cells that had been treated with 100 μM BH4 for 3 and 24 h. Data are expressed as the mean±SEM in percentage of control; *p$<$0.05, **p$<$0.01 vs. untreated control.

dramatic effect. At 100 μM BH4, the aconitase activity was reduced immediately within 15 min to 7.7% of untreated control (Fig. 1C). A significant decrease was also observed at 10 μM BH4 (Fig. 1D). Therefore, it appeared that the activity of the existing enzyme, rather than a change in gene expression, is involved in the BH4- induced reduction of aconitase activity. Because aconitase is present in the cytosol as well as in the mitochondria, we asked whether one form might be affected more than the other. For this, we fractionated the lysate of cells that had been exposed to BH4 into mitochondrial and cytosolic fractions and subjected each to aconitase activity assay. As shown in Fig. 1E, the activity of both forms was equally decreased by BH4.

The inactivation is partially irreversible

It has been reported that aconitase acts as a ROS sensor and that its inactivation by ROS is reversible. Therefore, we tested whether the activity reduced by the BH4 exposure might be restored by reactivation. For this, the lysate of cells treated with BH4 for 24 h was exposed to dithiothreitol, Na$_2$S and ferrous ammonium sulfate. The results showed that the enzyme activity was recovered to 87% of untreated control, from 66% before the reactivation (Fig. 2). Abou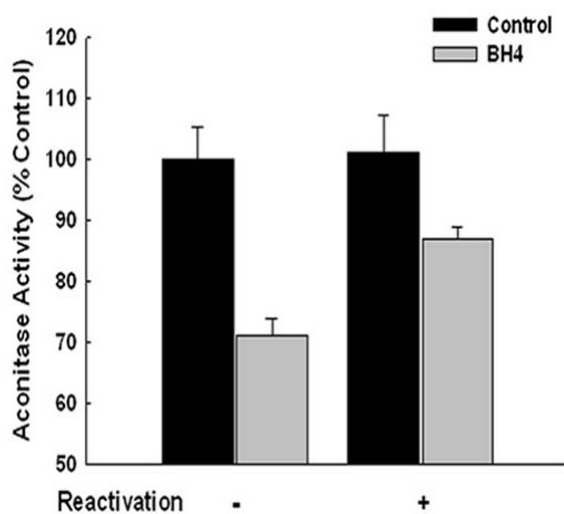t 34% of the activity reduced by the BH4 treatment could not be recovered, indicating that the BH4-induced inactivation was only partially reversible. This suggested that irreversible covalent modification might also have occurred.

DA is involved in the aconitase inactivation

We have previously observed that DA makes cells vulnerable, and that this is because in the presence of BH4, DA is readily oxidized to DA quinone. We therefore tested whether the addition of DA may exacerbate the effect of BH4 on aconitase. Cells were coincubated with 100 μM DA and 100 μM BH4, and the degree of inactivation was compared with that of cells treated with BH4 alone. As shown in Fig. 3, DA further lowered the aconitase activity from 66% to 45%, suggesting that DA amplified the inactivation. For further confirmation, we depleted the DA content in the cell with 100 μM α-methyl- p-tyrosine, the inhibitor of tyrosine hydroxylase, before exposing the cells to BH4. In this case, the aconitase inactivation was not as dramatic (88% of untreated control). Taken together, the presence of DA in DArgic cells appeared to contribute to inactivation of aconitase.
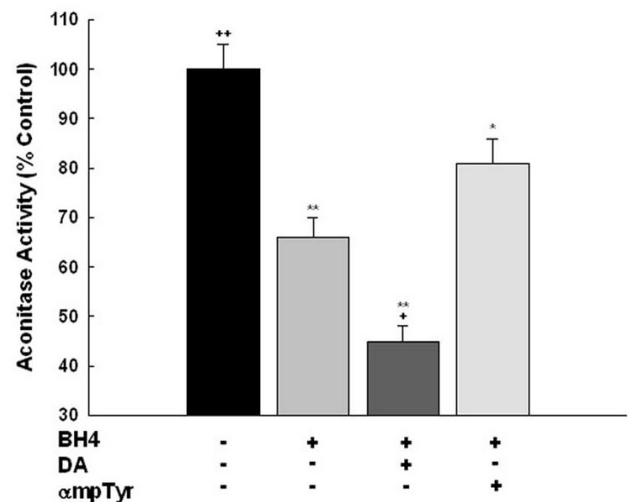

Fig. 2. Aconitase inactivation by BH4 is partially irreversible. The lysate of CATH.a cells that had been exposed to 100 μM BH4 for 24 h was obtained and incubated in reactivation solution. Aconitase enzyme activity was measured and expressed as the mean±SEM in percentage of control.

Fig. 3. DA is involved in the aconitase inactivation. Cells were treated with 100 μM BH4 for 24 h in the presence of dopamine (100 μM) or after a 24 h-pretreatment with 100 μM α-methyl-p-tyrosine. Aconitase enzyme activity was measured in the cell lysate and expressed as the mean±SEM in percentage of control; *p<0.05, **p<0.01 vs. untreated control: [+]p<0.05, [++]p<0.01vs. BH4-treated.
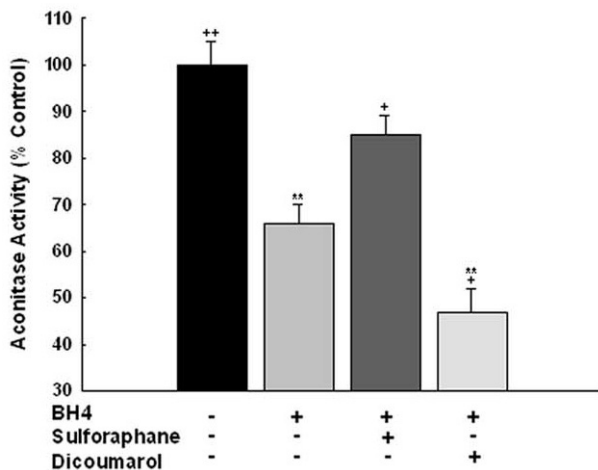
Fig. 4. DA quinone is involved in the aconitase inactivation. Cells were treated with 100 μM BH4 for 24 h after a 24 h-pretreatment with 2.5 μM sulforaphane or in the presence of dicoumarol (10 μM). Aconitase enzyme activity was measured in the cell lysate and expressed as the mean±SEM in percentage of control; *$p < 0.05$, **$p < 0.01$ vs. untreated control: [+]$p < 0.05$, [++]$p < 0.01$ vs. BH4-treated.

DA quinone is involved in the aconitase inactivation

Because the oxidation of DA to DA quinone is thought to potentiate generation of ROS in DArgic cells, we tested whether removal of DA quinone might attenuate the inactivation. For this, the cells were pretreated with sulforaphane, the compound known to elevate gene expression of quinone reductase, at conditions previously shown to induce quinone reductase in CATH.a cells (Han et al., 2007). Under this condition, the degree of inactivation was smaller (85% of untreated control; Fig. 4). We also tested whether inhibition of quinone reductase might have a worsening effect. Cotreatment of cells with the quinone reductase inhibitor dicoumarol (10 μM) resulted in further inactivation compared to treatment with BH4 alone (45% of untreated control).

DISCUSSION

A role of aconitase inactivation in neurodegenerative diseases has been suggested. Decreased aconitase activity is observed in various animal and cell models of neurodegeration including the MPTP model of PD (Liang and Patel, 2004), the DJ-1 mutant mouse model of PD (Andres-Mateos et al., 2007), the β-amyloid model of Alzheimer's disease

(Longo et al., 2000), and superoxide dismutase-2 mutant mouse model of amyotropic lateral sclerosis (Melov et al., 1999; Liang and Patel, 2004), as well as in aging (Yan et al., 1997). We demonstrate in the present study that BH4, the endogenous molecule that leads to DArgic cell demise (Choi et al., 2000; 2003), also causes a decrease in aconitase activity.

The mitochondrial aconitase catalyzes the reversible isomerization of citrate and isocitrate via its intermediate form, cis-aconitate, in the Kreb's cycle. The cytosolic aconitase is also known as iron regulatory protein 1, and acts as a transcription factor binding to iron-responsive elements, thereby inducing transcripts of genes involved in iron metabolism and/or energy metabolism (Rouault, 2006). We observed in the current study that both aconitases are inactivated after cellular exposure to BH4. As BH4 is known to facilitate production of superoxide radical (Fisher and Kaufman, 1973; Davis et al., 1988; Davis and Kaufman, 1993) and the iron-sulfur center of aconitases is susceptible to inactivation by superoxide radical (Gardner and Fridovich, 1992; Hausladen and Fridovich, 1994; Li et al., 2001), it is likely that the inactivation of aconitases by BH4 involves superoxide radical.

We also observed that the inactivation is only partially reversible. The aconitases are thought to act as an ROS sensor, inactivated in the presence of ROS but reactivated under normal condition. Therefore, the activity not recovered by the reactivation reaction would be due to irreversible inactivation. Indeed we have noted a shift in mobility of aconitase upon 2D gel electrophoresis, suggesting a covalent modification (data not shown). In DArgic neurons, DA can be auto-oxidized to form reactive quinone species capable of covalently modifying and damaging cellular macromolecules (Hastings et al., 1996). An elevated level of auto-oxidation products of cytoplasmic DA in the Parkinsonian substantia nigra has been noted (Fornstedt et al., 1989; Spencer et al., 1998). That aconitase is irreversibly inactivated by DA quinone is supported by the finding that induction of quinone reductase prevented aconitase inactivation and inhibition of quinone reductase amplified the inactivation.

The irreversibly inactivated aconitase may trigger a vicious cycle of producing hydrogen peroxide and

free iron, which would in turn contribute to further generation of DA quinone in DArgic cells. In addition, the inactivation of aconitase would interfere with the Kreb's cycle and thus the normal production of ATP and NADH. Therefore, irreversible inactivation of aconitase would ultimately lead to ATP depletion and cell death.

In summary, we show in the present study that in DArgic cells, oxidative stress leads to inactivation of the mitochondrial and cytosolic aconitases and that this involves formation of both ROS and DA quinone. The role of DA quinone in covalent and irreversible modification of aconitase in DArgic neurons may be a factor that renders these cells particularly vulnerable.

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