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D609 mediated inhibition of ATP synthesis in neural progenitor cells

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Abstract

D609 (Tricyclodecan-9-yl-xanthogenate) is an anti-oxidative molecule exhibiting antiproliferative and neuroprotective properties in a variety of cells. Previously we have shown that D609 decreased the proliferation of neural progenitor cells. In this study we examined the anti-oxidative property of D609 on neural progenitor cells isolated from the subventricular zone of the rat brain. Cellular oxidation was assessed by measuring the ATP content of the cells. Our results show that D609 decreased the ATP content of the neural progenitor cells by approximately 40% suggesting the possible inhibition of cellular metabolic activity. Cytochrome C oxidase (COX) also known as complex IV of the electron transport chain is a terminal enzyme involved in the oxidation of substrates resulting in the generation of energy required for the cellular activity. Therefore, regulating the activity of COX could interfere with the generation of ATP consequently affecting the proliferation of cells. Consistent with this hypothesis, we also observed a decline in the cytochrome C oxidase activity following the incubation of neural progenitor cells with D609. These results suggest that D609 could inhibit the activity of cytochrome C oxidase and subsequent ATP synthesis in the neural progenitor cells.

Keywords

D609; Oxidation; Cytochrome C oxidase

Introduction

Tricyclodecan-9-yl-xanthogenate (D609) appears to be a multifunctional compound exhibiting an inhibitory effect on proliferation [1, 2] inflammation [3] and oxidation [4-6] in a variety of cells. Cellular oxidation of substrates produces energy in the form of ATP in mitochondria. During this process, reactive oxygen species (ROS) are also generated causing damage to proteins [7], DNA [8] and membrane phospholipids [9-10] thus playing a major role in cell death and disease process. However the ROS are scavenged by the endogenous antioxidants, thereby preventing cell death.

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D609 was originally discovered as a potent inhibitor of phosphatidylcholine specific phospholipase C (PCPLC), which plays a major role in the survival and proliferation of cells [11-12]. An earlier study has shown that D609 is a powerful antioxidant which behaves like a glutathione mimetic drug [13]. Likewise, there is abundant literature documenting the anti-proliferative property of D609 in a variety of cells. We have shown that D609 can inhibit the proliferation of neural progenitor cells [14]. In spite of several reports on D609 induced cell cycle arrest, the mechanism of action remains obscure. Moreover, very limited information is available regarding the anti-oxidative function of D609 on neural progenitor cells. Understanding the mechanism of D609 on its potential targets would enable us to harness its neuroprotective and anti-proliferative properties as a therapy to treat pathological conditions like stroke and cancer both of which require ATP for survival and proliferation. In the present report, we investigated the anti-oxidative property of D609 by studying the cytochrome c oxidase activity and ATP content of neural progenitor cells. These studies were performed using *in vitro* cell culture model of neural progenitor cells isolated from adult rat brain.

Materials and Methods

Materials

Adult male spontaneously hypertensive rats (250-350g) were purchased from Charles River Laboratories. Neurobasal medium, B27 (without retinoic acid), recombinant human FGF-2 and antibiotic mixture were purchased from Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from the following suppliers: CellTiter-Glow reagent (Promega Inc. Madison, WI, USA); Cytochrome C Oxidase kit, Accutase, Heparin and Glutamine were from Sigma chemical company (St. Louis MO, USA).

Cell culture

Neural progenitor cells were generated from the rat brain as previously described [15-16]. All of the experimental procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. Briefly, the animals were anesthetized using isoflurane and the subventricular zone (SVZ) tissue was carefully dissected from the brain. The SVZ tissue was minced and digested with papain, calpain, dispase as described earlier [15-16]. The dissociated cells were cultured in neurobasal medium containing B27, FGF-2, (20 ng/ml), glutamine (2 mM), antibiotics, and heparin (2 µg/ml) for 5-7 days and the resulting neurospheres were dissociated using accutase for further experiments. All the experiments were performed between passages 3-20.

Reduction of MTS reagent by D609

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is a tetrazolium salt which is converted into a formazan product upon reduction. Therefore, we examined the ability of D609 in reducing MTS into a formazan product by measuring its absorbance at 490 nm. D609 (0-25-50-100 µM) was added to culture medium containing B27 and FGF2 in a 96 well plate. After 24 h incubation, 20 µl of MTS reagent (CellTite 96 AQueous Promega Madison, WI) was added to the medium, incubated at 37°C

for ~ 2 hr and the absorbance was measured at 490 nm. The same volume of medium without D609 was used as blank. Each individual experiment was repeated 6 times.

ATP measurement

The total ATP content was determined by using CellTiter-Glow assay kit (Promega) as described in the instruction manual. Neural progenitor cells (1×10^4 / 50 μ l) were cultured in an opaque 96 well micro titer plate for 24h under various experimental conditions as described in the figure legends. At the end of experimental period, the cells were equilibrated at room temperature (RT) for 30 min and mixed with equal volume of CellTiter-Glow reagent (50 μ l) on a shaker for 2 min followed by continued incubation for another 10 min at RT. The luminescence generated by ATP was measured in a luminometer (Veritas) using promega protocol and expressed as relative light units (RLU). Each individual experiment was repeated 8-10 times in quadruplicate and represented as mean $\pm$ SD.

Cytochrome C oxidase activity (Cox)

Cytochrome C oxidase was measured using the Cyt C oxidase assay kit (Sigma Chemical Company, USA) according to the product instruction manual with few modifications. The activity of cytochrome c oxidase was measured by its ability to oxidize the reduced ferrocytochrome C and continuously monitoring the activity by measuring the absorbance at 550 nm for 1 min. Briefly cells were homogenized in a buffer containing 20 mM Tris-HCl (pH 7.2), 0.25 M Sucrose, 40 mM KCl, and 2 mM EGTA. The homogenate (100 μ g/0.3ml) containing triton-x-100 (0.1% final concentration) was incubated on ice for 15 min prior to enzyme assay. Twenty five micrograms (25 μ g) of total homogenate was used for enzyme assay in the presence of reduced ferrocytochrome C as described in the manufacturer's protocol. Ferrocytochrome C was reduced using 0.1 M DTT (5 μ l/ml) for atleast 15 min and its absorbance were measured at 550 and 565 nm before performing the assay. The reduced cytochrome C was used if the ratio of 550/565 was between 10 and 15 to ensure proper reduction of the compound. Each experiment is repeated 5-10 times and the activity of the enzyme is normalized to equal amount of protein and expressed as units/ml.

Mitochondrial membrane potential

Neural progenitor cells were cultured on polyornithine/laminin coated coverslips in the presence or absence of D609 for 24 h. TMRE (tetramethylrhodamine, ethyl ester; final concentration 50nM) was added to neural progenitor cells and incubated for 20 min to load mitochondria with dye. The cells were also stained with Hoechst to label nucleus. At the end of incubation images were captured with Nikon fluorescence microscope at 20 $\times$ magnification.

Statistical analysis

Data is represented as mean $\pm$ SD of atleast 5-10 individual experiments performed in quadruplicate and analyzed by student's "t" test for comparing 2 groups or ANOVA supplemented by Bonferroni post hoc test for more than 2 groups. Values were considered significant at $P < 0.05$.

Results

Effect of D609 on MTS reagent

The anti-oxidative property of D609 was assessed by studying the ability of D609 to reduce the tetrazolium compound (MTS reagent) into formazan product. Our results show that D609 reduced the MTS reagent as a function of concentration (**Table-1**) as shown by an increase in the absorbance at 490nm.

Effect of D609 on ATP content

We quantified the ATP content of the neural progenitor cells by measuring the luminescence produced due to the hydrolysis of ATP in a luciferin-luciferase dependent reaction as relative light units (RLU). The amount of light produced is proportional to the quantity of ATP present in the cells. Our results show that B27 (RLU=2444731) increased the content of ATP (by approximately 10 fold) in the neural progenitor cells as compared to control (RLU=222037) or FGF2 (RLU=351441) treated cells and there was no synergistic effect of FGF2 on B27 induced ATP content (Fig. 1A). Our results also demonstrate that D609 decreased (40%) the content of ATP in the neural progenitor cells cultured in complete medium (B27 + FGF2) for 24 h (Fig.1B).

Effect of D609 on cytochrome c oxidase activity (Cox)

The addition of B27 to neural progenitor cells cultured in neurobasal medium substantially increased the activity of Cox, which is not significantly different when compared to cells supplemented with B27+FGF2 (Fig.2A). However, D609 (100 μ M) reduced the activity of cytochrome C oxidase by approximately ~50% in the neural progenitor cells after a period of 24 h in culture (Fig. 2B).

Effect of D609 on mitochondrial membrane potential

The proton gradient across mitochondrial inner membrane is measured by adding the mitochondrial dye TMRE which can enter the mitochondria but will leak out in depolarized mitochondria subsequently resulting in cell death. Our results show that 98% of cells retained the dye and there was no difference between the cells cultured in control and D609 treated cells (Fig.3).

Discussion

Generally the oxidation of substrates generates energy in the form of ATP and reactive oxygen species (ROS) as a byproduct. During the normal metabolic process, ROS are neutralized by endogenous anti-oxidative molecules like glutathione thereby keeping up the generation of energy while scavenging the ROS. However during high oxygen demand as observed during reperfusion following ischemia, the anti-oxidative mechanisms are unable to cope up with the generation of ROS resulting in enhanced cell death. Therefore using anti-oxidative therapy during such a process can decrease the overproduction of ROS and reduce cell death. Likewise in a normal cell decreasing the substrate oxidation can result in the production of less ROS and ATP. A decline in the ATP content can slow down the cellular process of proliferation resulting in the arrest of cell cycle. Thus anti-oxidative

molecules seem promising for both neuroprotection and cancer due to their role in metabolic process.

It appears that D609 being an anti-oxidative [5-6] molecule can decrease the oxidation and increase the reduction of substrates. Consistent with this hypothesis our current data illustrates that D609 increased the reduction of MTS compound in a dose dependent manner by converting the tetrazolium salt (MTS) into a formazan product which is measured at 490 nm. Perhaps this could explain the increased reduction of MTS in our previous study [17] prompting us to interpret an enhanced metabolic activity of the cells after one day in culture. The reduction of MTS by D609 was observed after 24 h in culture but not after 4 days, possibly due to the degradation of D609 in medium over time as reported by us recently [14].

Our previous dose response studies have shown a significant inhibitory effect of D609 on the proliferation of neural progenitor cells only at 100 μ M concentration with no effect at lower (5, 50 μ M) concentration [17]. Therefore, we used 100 μ M for all our experiments in this study to be consistent with our previous findings. Our current results show that D609 decreased the ATP content of the cells by ~40%. One reason for the decreased ATP content could be due to an increased degradation or decreased synthesis of ATP by the neural progenitor cells. It is interesting to note that D609 inhibited the FGF2 mediated proliferation [14] but FGF2 did not increase the ATP content rather B27 stimulated the synthesis of ATP in the neural progenitor cells (Fig. 2). Majority of ATP is formed in the mitochondria during electron transport chain (ETC). Cytochrome C Oxidase (Cox) is a terminal enzyme in ETC which transfers electrons to molecular oxygen generating proton gradient, which is essential for the generation of ATP. Because D609 is an anti-oxidative molecule, we hypothesize it to inhibit the activity of cytochrome c oxidase, which uses oxygen during the last step of ETC. Therefore, we measured the activity of Cox in cells treated with D609 and other growth supplements. Consistent with our ATP data, Cox activity is also upregulated (10 fold) in cells cultured in B27 as compared to control and is not significantly different from B27+FGF2. Moreover, D609 inhibited the Cox activity of neural progenitor cells by approximately 50%. These findings indicate that D609 might decrease the synthesis of ATP by inhibiting the activity of cytochrome C oxidase. Our current results are in line with previous observations demonstrating the decreased synthesis of ATP following the inhibition of Cytochrome c Oxidase [18-19].

Generation of ATP in the mitochondria is coupled to proton gradient in the inner mitochondrial membrane during respiration. Growth factors play an important role in providing oxidizable substrates to mitochondria which produce proton gradient across mitochondrial membrane (20). In agreement with this hypothesis our study shows that neural progenitor cells cultured in growth medium produced high ATP content. Therefore we measured mitochondrial membrane potential using TMRE dye and observed that D609 did not alter the mitochondrial membrane potential as detected by the lack of change in the TMRE staining. Data from these studies suggest that although ATP content is decreased by ~40% following D609 treatment the proton gradient is maintained keeping the cells viable. These results are consistent with our previous findings which established that D609 (100 μ M) did not induce cell death (14) after 24 h in culture.

Conclusions

Our results indicate that D609 can decrease the ATP content of neural progenitor cells by inhibiting the cytochrome c oxidase mediated generation of ATP in cells.

Acknowledgments

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References

1. Gusain A, Hatcher JF, Adibhatla RM, Wesley UV, Dempsey RJ. Anti-proliferative effects of tricyclodecan-9-yl-xanthogenate (D609) involve ceramide and cell cycle inhibition. *Mol Neurobiol*. 2012; 45(3):455–464. [PubMed: 22415444]
2. Abalsamo L, Spadaro F, Bozzuto G, Paris I, Cecchetti S, Lugini L, et al. Inhibition of phosphatidylcholine-specific phospholipase C results in loss of mesenchymal traits in metastatic breast cancer cells. *Breast Cancer Res*. 2012; 14(2):R50. [PubMed: 22429397]
3. Kennedy A, Martinez AK, Chung S, LaPoint K, Hopkins R, Schmidt SF, et al. Inflammation and insulin resistance induced by trans-10, cis-12 conjugated linoleic acid depend on intracellular calcium levels in primary cultures of human adipocytes. *J Lipid Res*. 2010; 51(7):1906–1917. [PubMed: 20154361]
4. Zhou D, Lauderback CM, Yu T, Brown SA, Butterfield DA, Thompson JA. D609 inhibits ionizing radiation-induced oxidative damage by acting as a potent antioxidant. *J Pharmacol. Exp. Ther*. 2001; 298(1):103–109. [PubMed: 11408530]
5. Sultana R, Newman S, Mohammad-Abdul H, Keller JN, Butterfield DA. Protective effect of the xanthate, D609 on alzheimer's amyloid beta-peptide (1-42) induced oxidative stress in primary neuronal cells. *Free Radic. Res*. 2004; 38(5):449–458. [PubMed: 15293552]
6. Perluigi M, Joshi G, Sultana R, Calabrese V, De Marco C, Coccia R, Butterfield DA. In vivo protection by the xanthate tricyclodecan-9-yl-xanthogenate against amyloid beta-peptide (1-42) induced oxidative stress. *Neuroscience*. 2006; 138(4):1161–1170. [PubMed: 16427207]
7. Hensley K, Hall N, Subramaniam R, Cole P, Harris M, Aksenov M, et al. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J. Neurochem*. 1995; 65:2146–2156. [PubMed: 7595501]
8. Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, Beal MF. Oxidative damage to mitochondrial DNA shows marked age dependent increases in human brain. *Ann. Neurol*. 1993; 34:609–616. [PubMed: 8215249]
9. Lewen A, Hillered L. Involvement of reactive oxygen species in membrane phospholipid breakdown and energy perturbation after traumatic brain injury. *J. Neurotrauma*. 1998; 15(7):521–530. [PubMed: 9674555]
10. Latchoumycandane C, Marathe GK, Zhang R, McIntyre TM. Oxidatively truncated phospholipids are required agents of TNF α induced apoptosis. *J. Biol. Chem*. 2012; 18:17693–17705. [PubMed: 22433871]
11. Cui Z, Houweling ZM, Chen MH, Record M, Chap DE, Vance F. A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in Chinese hamster ovary cells. *J Biol. Chem*. 1996; 271:14668–14671. [PubMed: 8663247]
12. Arsenault DJ, Yoo BH, Rosen KV, Rodgway ND. Ras induced upregulation of CTP: phosphocholine cytidyltransferase α contributes to malignant transformation of intestinal epithelial cells. *J. Biol. Chem*. 2013; 288(1):633–643. [PubMed: 23155050]
13. Ansari MA, Joshi G, Huang Q, Opii WO, Abdul HM, Sultana R, Butterfield DA. In vivo administration of D609 leads to protection of subsequently isolated gerbil brain mitochondria subjected to invitro oxidative stress induced by amyloid beta peptide and other oxidative stressors; relevance to Alzheimer's disease and other oxidative stress related neurodegenerative disorders. *Free Radic. Biol. Med*. 2006; 41(11):1694–1703. [PubMed: 17145558]

14. Kalluri H, Gusain A Dempsey RJ. Regulation of neural progenitor cell proliferation by D609: Potential role for ERK. *Mol. Neurobiol.* 2013; 47(2):782–789. [PubMed: 23275176]
15. Wachs FP, Couillard-Despres S, Engelhardt M, Wilhelm D, Ploetz S, Vroemen M,J, et al. High efficacy of clonal growth and expansion of adult neural stem cells. *Lab. Invest.* 2003; 83(7):949–962. [PubMed: 12861035]
16. Kalluri H, Vemuganti R, Dempsey RJ. Mechanism of insulin like growth factor I mediated proliferation of adult neural progenitor cells: role of Akt. *Eur. J. Neurosci.* 2007; 25:1041–1048. [PubMed: 17331200]
17. Kalluri H, Dempsey RJ. D609 inhibits the proliferation of neural progenitor cells. *NeuroReport.* 2010; 21:700–703. [PubMed: 20568314]
18. Alonso JR, Cardellach F, Lopez S, Casademont J, Miro O. Carbon monoxide specifically inhibits cytochrome c oxidase of human mitochondrial respiratory chain. *Pharmacol. Toxicol.* 2003; 93(3): 142–146. [PubMed: 12969439]
19. Pearce LL, Bominaar EL, Hill BC, Peterson J. Reversal of cyanide inhibition of cytochrome c oxidase by the auxiliary substrate nitric oxide: an endogenous antidote for cyanide poisoning? *J Biol. Chem.* 2003; 278(52):52139–45. [PubMed: 14534303]
20. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol. Cell Biol.* 2001; 21:5899–5912. [PubMed: 11486029]

Fig.1.

A: Neural progenitor cells were cultured in neurobasal medium (NB) containing various growth factors (B27 and/or FGF2) as shown for 24h and ATP content was measured as described in methods. B27 significantly increased the ATP content as compared to control or FGF2. Addition of FGF2 to B27 did not significantly enhance the content of ATP. * $P < 0.05$ as compared to control. *NS: no significant difference as compared to B27+FGF2. The data is represented as mean $\pm$ SD of NB (n=12), FGF2 (n=9), B27 (n=13), B27+FGF2 (n=11) individual experiments performed in quadruplicate and analyzed using ANOVA supplemented by bonferroni post hoc test.

B: Neural progenitor cells were cultured in complete medium (B27 + FGF2) with or without D609 (100 μ M) for 24h. D609 decreased the ATP content by ~40%. * $P < 0.05$. Data shown is represented as mean $\pm$ SD of 10 individual experiments performed in quadruplicate and analyzed using student's 't' test.

Fig. 2.

A: Neural progenitor cells were cultured in neurobasal medium (NB) or NB + B27 or NB + B27 + FGF2 as shown for 24h and cytochrome c oxidase (Cox) activity was measured using a spectrophotometer. B27 significantly increased the activity of Cox as compared to control but FGF2 did not show any synergistic effect on B27-induced Cox activity. * $p < 0.05$ as compared to control *NS: not significantly different as compared to B27+FGF2. Data is represented as mean $\pm$ SD of NB(5), B27 (6), B27+FGF2 (6) individual experiments analyzed using ANOVA supplemented by Bonferroni post hoc test.

B: Neural progenitor cells were cultured in complete medium (B27 + FGF2) with or without D609 (100 μ M) for 24h. D609 significantly decreased the activity of Cox by ~50%. $n=10$. * $P < 0.05$. The represented data is mean $\pm$ SD of 10 individual experiments and analyzed using student's 't' test. A unit of Cox activity is defined as the amount of enzyme that catalyzes the reduction of 1 μ mol of ferrocytochrome-c per minute at room temperature.

Fig. 3.

D609 did not alter the mitochondrial membrane potential. Neural progenitor cells were cultured on polyornithine/laminin coated coverslips in the presence and absence of D609 (100 μ M) for 24 h. At the end of experimental period TMRE & Hoechst were added to the cells to stain mitochondria and nuclei respectively. TMRE staining was similar between control and D609 treated cells. Red: TMRE Blue: Hoechst. n=3

Scale bar: 50 μ m.

Table. 1

Reduction of MTS reagent (at 490 nm) in the medium as a function of various concentrations of D609 (25-100 μ M).

Expt. Group	Mean $\pm$ SD
Control	0.154 $\pm$ 0.012
D609 (25 μ M)	0.173 $\pm$ 0.01 *
D609 (50 μ M)	0.202 $\pm$ 0.016 *
D609 (100 μ M)	0.261 $\pm$ 0.031 *

n=6 * P < 0.01 as compared with the control.