Mol Neurobiol (2010) 41:206–217
DOI 10.1007/s12035-010-8100-1

# Protection by D609 Through Cell-Cycle Regulation After Stroke

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Received: 18 November 2009 / Accepted: 8 January 2010 / Published online: 12 February 2010
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**Abstract** Expressions of cell-cycle regulating proteins are altered after stroke. Cell-cycle inhibition has shown dramatic reduction in infarction after stroke. Ceramide can induce cell-cycle arrest by up-regulation of cyclin-dependent kinase (Cdk) inhibitors p21 and p27 through activation of protein phosphatase 2A (PP2A). Tricyclodecan-9-yl-xanthogenate (D609)-increased ceramide levels after transient middle cerebral artery occlusion (tMCAO) in spontaneously hypertensive rat (SHR) probably by inhibiting sphingomyelin synthase (SMS). D609 significantly reduced cerebral infarction and up-regulated Cdk inhibitor p21 and down-regulated phospho-retinoblastoma (pRb) expression after tMCAO in rat. Others have suggested bFGF-induced astrocyte proliferation is attenuated by D609 due to an increase in ceramide by SMS inhibition. D609 also reduced the formation of oxidized phosphatidylcholine (OxPC) protein adducts. D609 may attenuate generation of reactive oxygen species and formation of OxPC by inhibiting microglia/macrophage proliferation after tMCAO (please also see note added in proof: D609 may prevent mature neurons from entering the cell cycle at the early reperfusion, however may not interfere with later proliferation of microglia/ macrophages that are the source of brain derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1) in offering protection). It has been proposed that D609 provides benefit after tMCAO by attenuating hypoxia-inducible factor-1α and Bcl2/adenovirus E1B 19 kDa interacting protein 3 expressions. Our data suggest that D609 provides benefit after stoke through inhibition of SMS, increased ceramide levels, and induction of cell-cycle arrest by up-regulating p21 and causing hypophosphorylation of Rb (through increased protein phosphatase activity and/or Cdk inhibition).

**Keywords** Cdk inhibitors · Cell-cycle inhibition · Ceramide · CNS injury · Focal cerebral ischemia · HIF-1α · Inflammation · Lipid peroxidation · PC-PLC · p21 · Phosphatidic acid · pRb · Sphingomyelin synthase · Stroke

**Abbreviations**

| | |
|---|---|
| ARE | Antioxidant response element |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| ASMase | Acid sphingomyelinase |
| BDNF | Brain derived neurotrophic factor |
| bFGF | Basic fibroblast growth factor |
| BNIP3 | Bcl2/adenovirus E1B 19kDa interacting protein 3 |
| CC | Contralateral cortex |
| CCT | Cytidine triphosphate (CTP):phosphocholine cytidylyltransferase |
| Cdk | Cyclin dependent kinase |
| CDP-choline | Cytidine 5′-diphosphocholine |

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|---|---|
| CPT | CDP-choline:DAG phosphocholine transferase |
| CTP | Cytidine triphosphate |
| D609 | Tricyclodecan-9-yl-xanthogenate |
| DAG | 1,2-diacylglycerol |
| EPO | Erythropoietin |
| GLUT-1 | Glucose transporter-1 |
| HIF | Hypoxia-inducible factor |
| IC | Ischemic cortex core |
| IGF-1 | Insulin-like growth factor |
| IL-1ß | Interleukin 1ß |
| IL-6 | Interleukin 6 |
| $IP_3$ | Inositol 1,4,5-trisphosphate |
| LPA | Lyso-phosphatidic acid |
| OxPC | Oxidized phosphatidylcholine |
| PA | Phosphatidic acid |
| PC | Phosphatidylcholine |
| PC-PLC | PC-phospholipase C |
| Penum | Penumbra |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| PKC | Protein kinase C |
| $PLA_2$ | Phospholipase A2 |
| PLD | Phospholipase D |
| PP | Protein Phosphatase |
| pRb | phospho-Retinoblastoma |
| Rb | Retinoblastoma |
| ROS | Reactive oxygen species |
| SM | Sphingomyelin |
| Smase | Sphingomyelinase |
| SMS | Sphingomyelin synthase |
| SPT | Serine palmitoyltransferase |
| TNF-α | Tumor necrosis factor-α |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| VEGF | Vascular endothelial growth factor |
| VHL | von-Hippel Lindau protein |

## Introduction

Expressions of cell-cycle regulating proteins are altered after stroke [1, 2]. In dividing non-neuronal cells such as astrocytes, microglia/macrophages, activation of the cell-cycle machinery induces proliferation. However, in post-mitotic neurons, cell-cycle entry results in their death [2, 3]. Cell-cycle inhibition has shown dramatic reduction in infarction after stroke [4]. Phosphatidylcholine (PC)-phospholipase C (PC-PLC) hydrolyzes PC to release 1,2-diacylglycerol (DAG) and phosphocholine. This reaction is distinct from phosphatidylinositol 4,5-bisphosphate ($PIP_2$) hydrolysis by phosphatidylinositol (PI)-PLC to release DAG and inositol 1,4,5-trisphosphate ($IP_3$). The primary intracellular target for DAG is activation of protein kinase C (PKC) which stimulates cell proliferation [5]. Sphingomyelin (SM) synthase (SMS) transfers the phosphocholine group from PC to ceramide to form SM and release DAG. Inhibition of PC-PLC or SMS will preserve PC and limit DAG release. PC-PLC and SMS have similarities in that both enzymes use PC as a substrate and produce DAG, thus it has been suggested that SMS may account for the putative PC-PLC [6].

Tricyclodecan-9-yl-xanthogenate (D609) is a SMS/PC-PLC inhibitor [6, 7] with anti-viral, anti-tumoral actions [7], and antioxidant/glutathione mimetic properties [8, 9] due to the presence of the thiol function. GSH mimetic/antioxidant actions are not based on stroke models [10]. The xanthate group of D609 can readily oxidize to form a disulfide [11], which is a substrate for glutathione reductase, regenerating D609 [10]. Pro-drug modifications of D609 may increase D609's stability since half-life of D609 in saline is short (~20 min) [11]. Actions of D609 attributed to PC-PLC inhibition include suppressing expression of hypoxia-inducible factor 1α (HIF-1α) after stroke [12], reduced cytokine expression in lipopolysaccharide (LPS)-stimulated macrophages [13, 14], prevention of tumor necrosis factor-α (TNF-α) or LPS-induced lethal shock in mice [15], and protection of immature neurons from oxidative glutamate toxicity (5 mM glutamate) by uncoupling the cystine/glutamate anti-porter [16]. Studies indicated that inhibition of PC-PLC by D609-enhanced phospholipase D (PLD) activity in UMR-106 osteoblastic cells either due to a compensatory effect or that D609 directly increased PLD activity [17]. D609 (375 μM) did not inhibit TNF-α-stimulated phospholipase $A_2$ ($PLA_2$) activity [18], but inhibited arachidonic acid release by $cPLA_2$ in MDCK cells at 750 μM [19]. D609 inhibited bFGF-stimulated astrocyte proliferation, which was attributed to SMS inhibition and increase in ceramide [20]. There are two forms of SMS (SMS1 in Golgi and SMS2 on the plasma membrane) and D609 inhibits both forms [21]. Through SMS inhibition and increased ceramide [6, 22], D609 can induce cell-cycle arrest by up-regulating cyclin dependent kinase (Cdk) inhibitors p21 and p27 [22]. c-Myc suppresses expression of p21 [23] and p27 [24], and ceramide can induce expression of p21 and p27 through activation of protein phosphatase 2A (PP2A) that down-regulates c-Myc [25, 26]. Collectively, these studies suggested that D609 could provide neuroprotection by blocking the cell cycle in post-mitotic neurons.

Mammalian cells have evolved a complex physiological response to reduced oxygen availability (hypoxia) in an attempt to prevent energy failure and maintain production of ATP. Many of these physiological responses are regulated by a family of transcription factors denoted hypoxia-inducible factors (HIF), heterodimeric transcription factors consisting of α and ß subunits [27, 28]. HIF-1ß, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) is constitutively expressed. Three mammalian α-subunits have been identified, HIF-1α, HIF-2α (EPAS), and HIF-3α, which are all oxygen sensitive but differ in their tissue expression. HIF-

1α is also constitutively expressed but is rapidly degraded under normoxic conditions by ubiquitin-proteosome pathway (Fig. 1). HIF-1α stability is regulated by hydroxylation of two proline residues by the oxygen-dependent HIF prolyl-4-hydroxylases. Hydroxylation of HIF-1α regulates its interaction with von-Hippel–Lindau (VHL) protein. VHL organizes the assembly of a complex that activates the E3 ubiquitin ligase, which then ubiqitinylates HIF-1α, targeting its degradation. Under hypoxic conditions, HIF-1α is stabilized and hetero-dimerizes with HIF-1ß. Thus, HIF-1α serves as the oxygen-sensing subunit of HIF-1. HIF-1 binds to hypoxia response elements (HRE) in the promoters, up-regulating its target genes, including vascular endothelial growth factor (VEGF), glucose transporters (GLUT), erythropoietin (EPO), and Bcl2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3).

HIF-1α is induced after cerebral ischemia [29, 30]. In reperfusion models, with tissue re-oxygenation, it would be expected that HIF-1α would be degraded due to re-activation of oxygen-dependent prolyl hydroxylases. HIF1 can be activated by factors other than hypoxia, such as interleukin-1ß (IL-1ß), TNF-α, transforming growth factor-ß (TGF-ß), insulin, and insulin-like growth factor 1 (IGF1) [28]. In one study, prolonged HIF-1α expression after tissue re-oxygenation in transient cerebral ischemia was attributed to up-regulation of IGF1 [30].

PC-PLC releases DAG from PC. DAG can be phosphorylated by DAG kinases to phosphatidic acid (PA). Induction of HIF-1α after transient middle cerebral artery occlusion (tMCAO) may be mediated by PA formed from DAG by the action of DAG kinase [31, 32], although the mechanism whereby PA activates HIF-1α has not been elucidated [32]. Activation HIF-1α by PA could be mediated through lyso-PA (LPA), which activates HIF-1α [33]. Recent studies [12] have proposed that D609 provides benefit after stroke by attenuating HIF-1α and BNIP3 expressions through inhibition of PC-PLC/SMS and release of DAG [31, 32]. The neuroprotective role of HIF-1α in various CNS disorders and injuries has been reviewed [34]. However, the role of HIF-1α in ischemic injury remains a matter of controversy [29, 35], since HIF-1 can mediate both pro- and anti-apoptotic effects [36] (Fig. 1) and neuroprotective mechanisms of D609 need re-examination. Recent studies showed in in vitro ischemia that inhibition of HIF-1α causes a drop in redox environment, which worsens cell injury [37]. Phosphorylation of HIF-1α promotes its binding to HIF-1ß and transcription of VEGF, GLUT-1 and EPO that promote survival. HIF-1 also participates in hypoxia-induced cell death by up-regulating transcription of BNIP3. In addition, under severe hypoxic conditions, non-phosphorylated HIF-1α binds to p53 and up-regulates pro-apoptotic Bax [36]. HIF-1 transcriptional activities may be dependent on ischemic severity, operated through p300-dependent and independent pathways. Whether HIF-1-mediated pro-survival or pro-death signaling predominates after stroke may depend on the ischemic duration, with HIF-1 exerting detrimental effects after more severe ischemia [12, 36].

Our results support the view that D609 inhibition of SMS and increase in ceramide levels induce expression of the

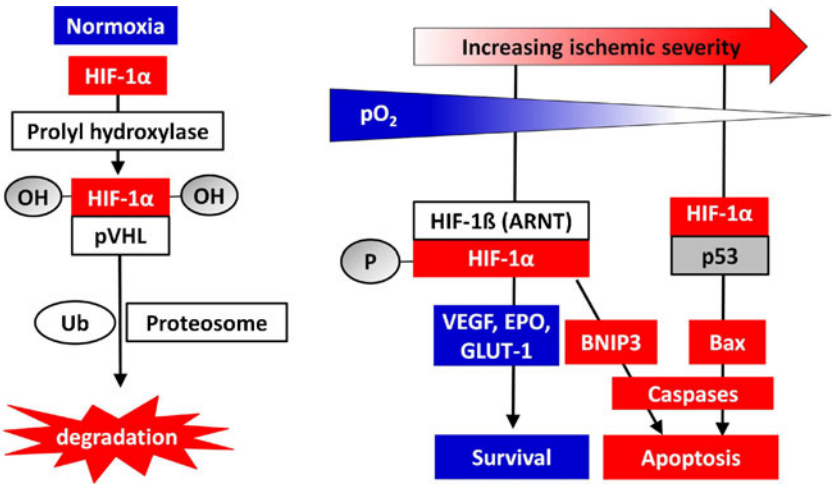


**Fig. 1** Under normoxic conditions hypoxia-inducible factor-1α (HIF-1α) is rapidly degraded by an ubiquitination pathway by the actions of $Fe^{2+}$, prolyl hydroxylases, tumor suppressor von Hippel–Lindau protein (*VHL*). Under hypoxic conditions prolyl hydroxylases are inactive, HIF-1α is stabilized, gets phosphorylated and translocates to nucleus where it complexes with HIF-1ß (*ARNT*), binds to hypoxia response elements (*HRE*) and initiates gene transcription, up-regulating pro-survival/adaptive (*VEGF*, *EPO*, and *GLUT-1*), and pro-apoptotic Bcl2/adenovirus E1B 19 kDa interacting protein 3 (*BNIP3*) genes. Under severe hypoxic conditions, non-phosphorylated HIF-1α binds to and stabilizes p53, up-regulates Bax, and induces apoptosis [36]. Subsequent caspase activation can cleave HIF-1ß, switching off HIF-1 signaling, and promoting further dephosphorylation of HIF-1α. In stroke, HIF-1 signaling may shift from pro-survival to pro-apoptotic with increasing ischemic severity

endogenous Cdk inhibitor p21, leading to hypophosphorylation of retinoblastoma (pRb) protein and inhibition of the cell cycle. Lipid metabolites that affect the cell-cycle system in stroke have not been extensively studied. These studies explore the therapeutic potential of D609 and how it affects lipid second messenger ceramide that regulates the cell cycle in a stroke model and a mechanism of action of D609 has been proposed. Our study as well as studies by others has shown neuroprotection by D609, however the routes of action appear to be different; these comparisons have been summarized and possible explanations have been offered.

## Materials and Methods

### Materials

All chemicals and reagents unless stated otherwise were purchased from Sigma (St. Louis, MO). D609 was obtained from Kamiya Biomedical Company (Seattle, WA) and ENZO/BioMol (Plymouth meeting, PA). The following antibodies were obtained from the indicated suppliers: ASMase, PKCζ (Santa Cruz Biotech, Santa Cruz, CA), p21 (BD Biosciences, San Diego, CA); p27, pERK, pAKT, Cyclin D1, phospho-Rb (Cell Signaling, Danvers, MA), horseradish peroxidase conjugated goat anti-rabbit and goat anti-mouse IgG (Bio-Rad, Hercules, CA) and donkey anti-goat IgG (Santa Cruz). EO6-monoclonal antibodies, which recognize oxidized PC (OxPC) as well OxPC bound proteins, were generously provided by Dr. Witztum, University California-San Diego. Detection of Western blots used SuperSignal from Pierce (Rockford, IL).

### Focal Cerebral Ischemia

All surgical procedures were conducted according to the animal welfare guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press Washington, D.C. 1996) and were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. These studies used tMCAO in spontaneously hypertensive rats (SHR); we [38, 39] and others [40] have shown that SHR provide a consistent infarction volume with a low variability. The coefficient of variation in the injury volumes is much less in SHR compared to Sprague–Dawley rats [39]. Male SHR (250–300 g) were purchased from Charles River (Wilmington, MA) and subjected to 1 hr tMCAO as described earlier [38, 41, 42]. Under isoflurane (1–2%) in an $O_2$ and $N_2O$ (50:50) mixture, a 3–0 monofilament nylon suture was introduced through the left internal carotid to occlude the middle cerebral artery. Reduction in blood flow was confirmed using a laser Doppler blood perfusion monitor (Vasamedics, LLC, St. Paul, MN). After 1 h occlusion, the suture was withdrawn to restore the blood flow that was confirmed by laser Doppler flowmetry. Mean arterial blood pressure, blood gases $PaO_2$ and $PaCO_2$ were monitored via a catheter inserted into the left femoral artery. Body temperature was maintained at 37–38°C by means of a thermostatically controlled water blanket.

### Drug Treatment

D609 (50 mg/kg i.p.) was dissolved in saline and administered at the onset of reperfusion. Controls received a corresponding volume of saline. Treatment with D609 did not affect the physiological variables and did not cause any hypothermia [12].

### Ischemic Injury (Infarction) Volume

Infarction volumes were measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining as described previously [38, 39, 41, 42]. Brains were cut in 2 mm coronal slices, incubated with 2% TTC for 30 min at 37°C, rinsed with saline and fixed in 4% paraformaldehyde. Stained sections were scanned and the ischemic injury volumes were computed by the numeric integration of data from individual slices using ImageJ program (freeware from NIH). To compensate for edema in the ischemic hemisphere, corrected infarction volumes were calculated as: corrected infarction volume=right hemisphere volume—(left hemisphere volume—measured infarction volume) [43].

### Brain Tissue Collection

For lipid analyses and Western blotting, brains of anesthetized rats were *in situ* frozen, dissected at 0°C, and brain sections were stored at −80°C until analyzed.

### Western Blot Analyses

Brain tissue was homogenized in lysis buffer (20 mM $Na_2HPO_4$, 50 mM NaF, 10 mM $Na_4P_2O_7$, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 2% Triton X-100, 0.5% deoxycholate; $Na_3VO_4$ (1 mM), and Sigma protease inhibitor cocktail were added immediately prior to use). For OxPC analyses, 20 μM BHT was added to the lysis buffer to prevent oxidation during sample preparation. Five to fifty μg of protein was loaded onto polyacrylamide gels. SDS-PAGE was performed using the Criterion system (Bio-Rad) at a constant voltage of 150 V. Proteins were subsequently transferred to nitrocellulose at a constant voltage of 100 V for 1 hr. Non-specific binding sites were blocked with 5% non-fat powdered milk in 1x Tris buffered saline (TBS) with 0.05% Tween-20 (1x TBST) at room temperature for 1 h. Blots were incubated with primary antibodies (diluted in either 5% BSA or 5% non-fat powdered milk in 1x TBST)

for overnight at 4°C, washed with 1x TBST, then incubated with appropriate secondary antibodies for 1 hr at room temperature. After washing, protein bands were visualized with SuperSignal and exposure to X-ray film.

### Lipid Analysis

All solvents and extracts were purged with $N_2$ during the extraction, TLC and methylation of lipids. Total lipids were extracted from brain tissue into $CHCl_3$/MeOH (1:2 by volume) containing 0.01% BHT, separated by TLC, converted to methyl esters and quantitated using a Hewlett Packard 6890 gas chromatograph as described earlier [44, 45].

### Statistical Analyses

Data were presented as mean±SD ($n$=3–4 per group) and analyzed by ANOVA followed by Bonferroni's multi-group comparisons post-test using Prism software (GraphPad, San Diego, CA). A value of $p<0.05$ was considered significant.

## Results

### D609 Attenuated Infarction After tMCAO

D609 (50 mg/kg i.p. in saline) given at the onset of reperfusion reduced infarct volume by 35±5% and 60±10% on days 1 and 3, respectively (1-day saline 270±38 $mm^3$; D609 176±9 $mm^3$; 3-day saline 216±25 $mm^3$; D609 86±9 $mm^3$) (Fig. 2). Administration of D609 delayed by 3 h after initiation of reperfusion did not significantly reduce infarction.

### Effect of D609 on Cell Signaling and Cell-Cycle Proteins after tMCAO

Changes in protein expression following 1-h tMCAO were compared over 1–3 days reperfusion. ASMase expression was not changed in the ischemic cortex core (IC) or penumbra (Penum.) compared with contra-cortex at 1-day reperfusion, but showed a significant decrease with D609 treatment. p21 expression was undetectable in the contra-cortex after 1-day reperfusion and showed low levels in the IC and penumbra. D609 treatment resulted in a dramatic increase in p21 expression in the IC at 1 day but did not induce p21 in either the penumbra or contra-cortex. Expression of pERK1/2 was significantly increased in the penumbra, with a small increase in the IC, and was not significantly altered by D609. Expression of p27 showed a small but not significant decrease in the IC and penumbra compared with contra-cortex at 1-day reperfusion, and was not significantly altered by D609 treatment (Fig. 3). Cyclin D1, PKCζ, and pAKT expressions were unchanged following tMCAO and 1-day reperfusion and were not altered by D609.

Over 3 days reperfusion, p21 expression remained barely detectable in the contra-cortex, increased in the IC and was further up-regulated with D609 treatment (Fig. 4). At this time it is unclear whether this transient induction of p21 in ipsilateral cortex of saline rat at 2 day reperfusion after tMCAO is sporadic; however the treatment with D609 further increased the p21 that is consistent over the 3-day reperfusion period. pRb expression was unchanged in the IC following 1-day reperfusion but was significantly down-regulated by D609. On days 2 and 3, pRb expression was significantly reduced in the IC and remained low following D609 treatment. Contra-cortex expression of pRb did not

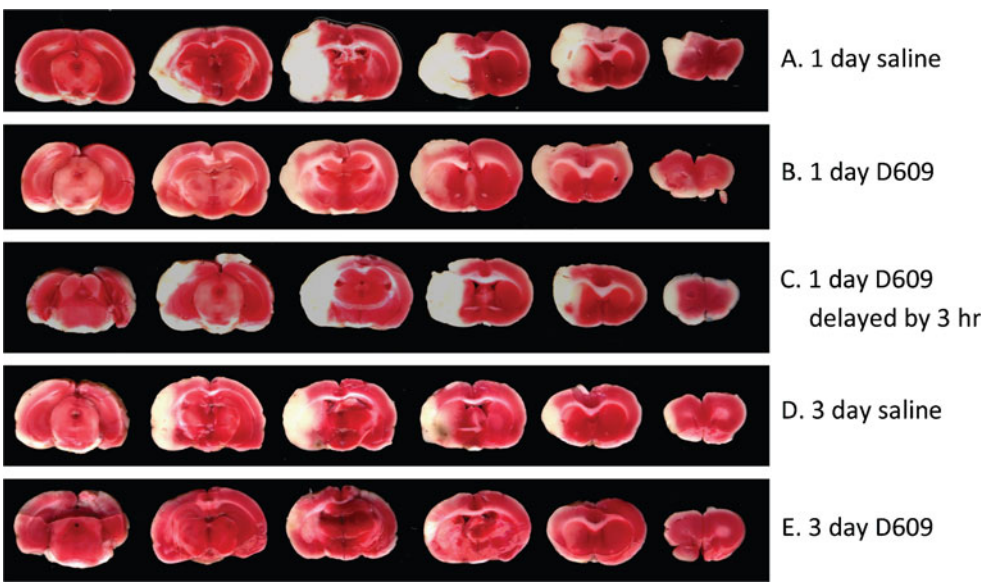


**Fig. 2** D609 significantly reduced the cerebral infarction volume after 1-h tMCAO and reperfusion at 1- and 3-day reperfusion compared with the respective saline-treated ischemic groups ($n$=3 per group). Saline vehicle (**a**, **d**) or D609 (50 mg/kg i.p.) was administered as a single dose at the onset of reperfusion (**b** and **e**) or after 3-h reperfusion (**c**). D609 reduced infarction by 35±9% (1-day rep.) and 65±15% (3-day rep.). D609 treatment delayed by 3 h did not reduce the infarction (**c**). There is reduction in infarction volume in saline-treated groups from 1- to 3-day by 19±8%

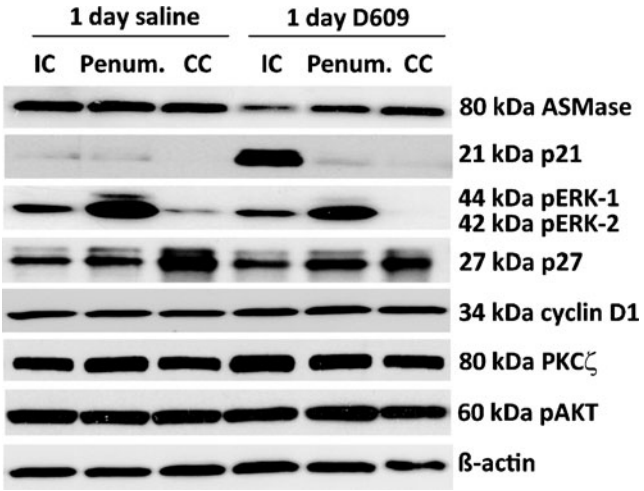


**Fig. 3** Western blots of acidic sphingomyelinase (ASMase), pERK1/2, p21, p27, cyclin D1, PKCζ, pAKT following 1-h tMCAO and 24-h reperfusion in SHR. PC-PLC inhibition may reduce DAG and subsequent ASMase expression. DAG regulates ASMase expression. *IC* ischemic cortex core; *Penum.* Penumbra; *CC* contralateral cortex

change over 3 days and was not affected by D609. The *loss* of pRb in the IC showed a good correlation with *induction* of p21 over 3 days reperfusion (Fig. 4). p27 expression showed no significant changes in IC compared with contra-cortex and was not altered by D609 treatment.

## Effect of D609 on Lipids After tMCAO

After tMCAO and 1 day reperfusion, there was a significant loss of PC and SM in IC compared to contra-cortex (Fig. 5a, b). Significant differences between groups are indicated in the respective graphs with connecting lines with the *p* values. D609 did not significantly affect PC or SM levels (Fig. 5a, b), but caused a significant increase in ceramide levels at 1 day compared with IC/saline treatment (Fig. 5c). At 3 days reperfusion, PC in the IC returned to contra-levels, however, remained low with D609 treatment (significantly below the 3 day levels in the IC/saline treated, Fig. 5a). SM levels in the IC at 3 days showed a significant decline with D609 treatment (compared to 3 day IC/saline,

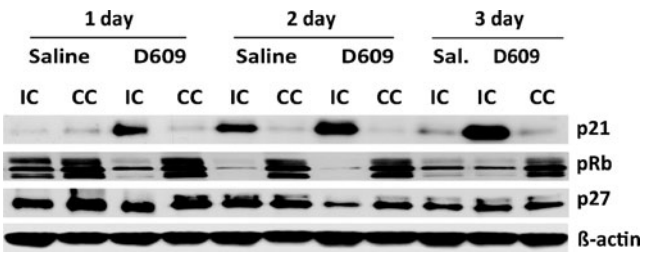


**Fig. 4** Western blot of p21, pRb, and p27 over 3 days of reperfusion following 1-h tMCAO in SHR. *IC* ischemic cortex core, *CC* contralateral cortex

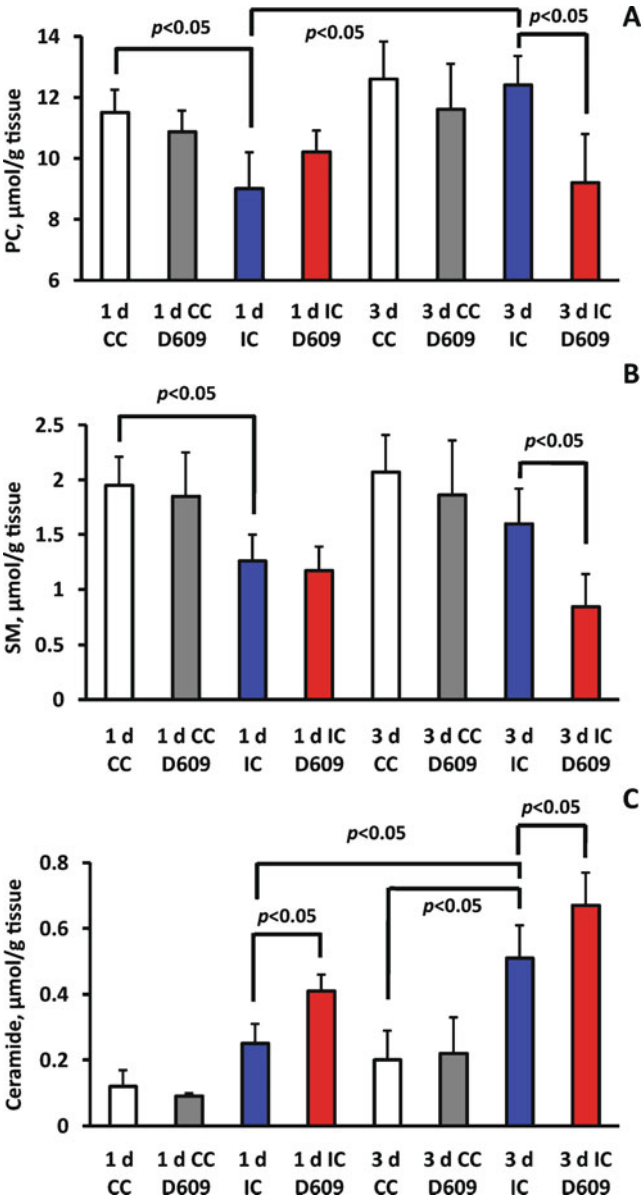


**Fig. 5** Effect of D609 on lipids after 1-h tMCAO/1- and 3-day reperfusion. *IC* ischemic cortex core, *CC* contralateral cortex. *PC* phosphatidylcholine, *SM* sphingomyelin; ($n$=4). Significant differences between groups are illustrated with connecting lines and the *p* values

Fig. 5b). At 3 days, ceramide levels significantly increased in the IC/saline treated (compared with 1 day IC) and showed a further increase with D609 treatment (3 day IC, D609 *vs* saline, Fig. 5c). D609 did not significantly alter contra-levels of PC, SM or ceramide at 1 and 3 days reperfusion.

## D609 Attenuated Formation of OxPC Protein Adduct(s) after tMCAO

OxPC protein adduct has been recognized in multiple sclerosis brains using EO6 antibodies [46]. Western blot

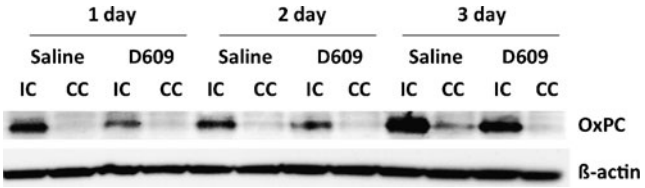


**Fig. 6** Western blot of OxPC protein adduct over 3 days of reperfusion following 1-h tMCAO in SHR. *IC* ischemic cortex core, *CC* contralateral cortex

analyses showed OxPC protein adduct increased in the IC over 3 days reperfusion, which was attenuated by D609 treatment (Fig. 6). The absence of OxPC in the contra-cortex suggests that its detection in the IC is not an artifact. Presently the identity of the OxPC-modified protein(s) is unknown [46]. Our observation is also supported by the recent observation by Hazen's group that showed novel OxPC species formation after permanent MCAO in mice [47]. Dot blot analyses did not show detectable levels of free OxPC in lipid form in either IC or contra-cortex after 1-h tMCAO and 24-h reperfusion [48], indicating that OxPC is predominantly in the protein-bound adduct.

## Discussion

D609 at the onset of reperfusion attenuated infarction at days 1 and 3, consistent with other studies showing neuroprotection by D609 [12, 49]. When treatment with D609 was delayed by 3 h, no significant reduction in infarct was observed. In other studies [12], D609 administered at 1-h reperfusion after 2-h MCAO significantly reduced infarct at 7 days reperfusion. These studies suggest that treatment with D609 during early reperfusion is critical in offering neuroprotection. Preliminary studies indicated that D609 (100 μM; $IC_{50}$ 94 μM [7]) increased the metabolic activity (MTT assay, a measure of mitochondrial function) of RAW 264.7 macrophages (data not shown), suggesting this drug may affect initial energy loss after ischemia, but further studies are needed to elucidate these aspects.

Our studies demonstrated increased ceramide levels in the IC after tMCAO, which became significant at 3 days, and D609 administered at the onset of reperfusion caused a significant increase in ceramide levels at 1 and 3 days reperfusion. Ceramide is released by ASMase, which can be activated by PC-PLC and formation of DAG [18]. Even though D609 attenuated ASMase expression at 1 day reperfusion, this did not result in a decrease in ceramide. This suggests that ceramide increases due to inhibition of SMS activity by D609 [6, 20, 21] offset any decrease in ceramide due to down-regulation of ASMase. Significant loss of SM and increase in ceramide was also observed in a model of permanent focal cerebral ischemia [50]. The increase in ceramide could be due to SMS inhibition [20], preventing incorporation of ceramide into SM. The increase in ceramide and loss of SM (D609 treatment *vs* saline) suggests that D609 inhibited SMS after tMCAO. Alternatively, it has been reported that D609 increased ceramide biosynthesis *in vitro* by stimulating activity of serine palmitoyltransferase (SPT) [51], the rate-limiting enzyme in ceramide *de novo* synthesis. However, those studies used a high concentration of D609 (375 μM) and it is unknown whether D609 affected SPT activity in increasing ceramide levels after tMCAO.

We investigated by Western blotting the expression of several proteins that have been shown to be involved in pathways of ceramide signaling (Fig. 7). Ceramide can bind to and activate PKCζ (an atypical PKC with no requirement for either $Ca^{2+}$ or DAG [52]). PKCζ in turn can activate the MEK/ERK pathway through Raf-1. Phosphorylated ERK1/2 (pERK1/2) accumulates in the nucleus where they phosphorylate transcription factors and up-regulate cyclin D1 expression. Cyclin D1 localizes to the nucleus, binds to Cdk4/6 and phosphorylates Rb and Rb-related proteins, allowing release of E2F transcription factors from their complex with Rb. E2F transcription factors regulate genes that are required for progression through $G_1$ phase and the $G_1$-S transition of the cell cycle.

In addition to activation of the PKC/ERK pathway, ceramide can also activate PI3 kinase (PI3K) [53], which phosphorylates phosphatidylinositol (PI) 4,5-bisphosphate

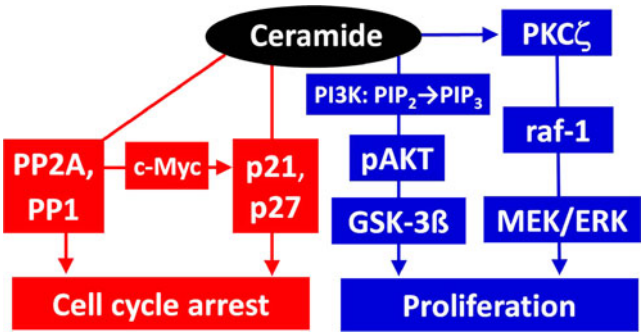


**Fig. 7** Ceramide regulation of cell proliferation and cell-cycle arrest. Cell proliferation: Ceramide can regulate cell proliferation through PKCζ, MEK/ERK, and pAKT pathways up-regulating cyclin D1. Ceramide can activate PI3 kinase (PI3K) to phosphorylate phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to $PIP_3$. $PIP_3$ is necessary for AKT to anchor to the plasma membrane for phosphorylation by PI-dependent kinase PDK1. When GSK-3ß is active, cyclin D1 is phosphorylated and exported from the nucleus for degradation by the ubiquitin-dependent 26S proteosome. pAKT phosphorylates and inactivates GSK-3ß, stabilizing cyclin D1. Ceramide also binds to and activates PKCζ, which activates MEK/ERK via raf-1. ERK1/2 then activate transcription factors and up-regulate cyclin D1 expression. Cell-cycle arrest: alternatively, ceramide can induce cell-cycle arrest through activation of PP1 and PP2A, dephosphorylation of pCdk2, up-regulating endogenous Cdk inhibitors p21 and p27, resulting in hypophosphorylation of pRb

($PIP_2$) to phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$). $PIP_3$ is necessary for anchoring AKT to the plasma membrane, allowing its phosphorylation (pAKT) by the PI-dependent kinase PDK1. $PIP_3$ is also required for recruitment and membrane binding of PDK1. Cyclin D1 stability and localization is regulated by glycogen synthase kinase-3*ß* (GSK-3*ß*). When GSK-3*ß* is active, cyclin D1 is phosphorylated and exported from the nucleus to the cytoplasm, where it is degraded *via* the ubiquitin-dependent 26S proteosome [54, 55]. pAKT phosphorylates and *inactivates* GSK-3*ß*, thus preventing cyclin D1 phosphorylation and nuclear export.

Since ceramide, acting through PKCζ → pERK1/2 and PI3K → pAKT pathways, could increase and stabilize cyclin D1 expression, we investigated the expression of pERK1/2, pAKT, and cyclin D1 after 1-h tMCAO and 1-day reperfusion. Consistent with the finding that expression of PKCζ and pAKT were unchanged, we did not observe any significant change in cyclin D1 expression after tMCAO or with D609 treatment (Fig. 3). Thus, the increase in ceramide did not alter these pathways, at least at the level of protein expression.

Ceramide signaling is complex and concentration dependent [56]: at low concentrations, ceramide can stimulate cell proliferation [57], whereas intermediate levels can inhibit cell proliferation [20], and apoptosis is induced at high levels [58]. Dose-dependent neuroprotective roles of ceramide have been demonstrated: exogenous ceramide (0.1–1.0 μM) protected hippocampal neuronal cultures against excitotoxicity, oxidative injuries and Aß toxicity [59], and at similar concentrations protected mesencephalic neurons from glutamate neurotoxicity [60]. Ceramide also prevented neuronal apoptosis induced by NGF deprivation [61]. Cell permeable exogenous ceramide reduced the infarction after MCAO in SHR [62]. Higher levels of ceramide become detrimental for cell survival [59, 60] as apoptotic pathways are induced.

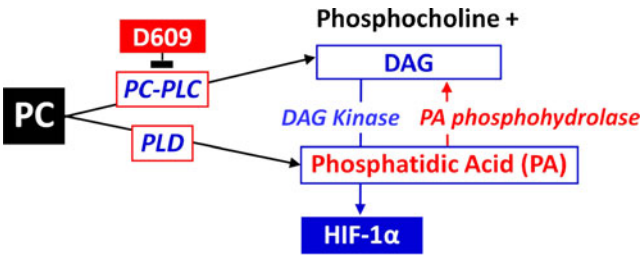


**Fig. 8** PC hydrolysis by PC-PLC, PLD, and formation of PA from DAG. D609 inhibits PC-PLC, attenuating DAG release and subsequent PA formation [12]. PA can be directly formed via PC hydrolysis by PLD. Inhibition of PC-PLC by D609 enhanced PLD activity either due to a compensatory mechanism or a direct increase in PLD activity by D609 [17]. Activation HIF-1α by PA could be mediated through lyso-PA [33]. *ASMase* acidic sphingomyelinase, *PA* phosphatidic acid, *PC* phosphatidylcholine, *PC-PLC* PC-phospholipase C, *PLD* phospholipase D. Enzymes are indicated by *italics*

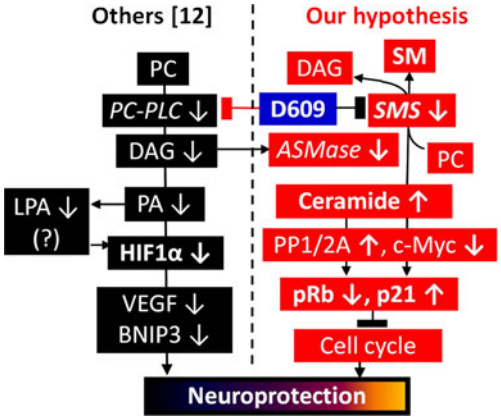


**Fig. 9** D609 offered protection in stroke models and here we present two possible routes of neuroprotection proposed through HIF-1α/BNIP3 [12] and through ceramide and cell-cycle arrest based on our present studies. Inhibition of PC-PLC may decrease DAG levels which subsequently reduce PA and HIF-1α expression. Activation HIF-1α by PA could be mediated through lyso-PA [33]. Our proposed mechanism of D609 action is operated through SMS inhibition that will cause accumulation of ceramide levels. Ceramide through activation of protein phosphatases PP1 and PP2A will increase p21 expression and hypophosphorylation of pRb. BNIP3: Bcl2/adenovirus E1B 19 kDa interacting protein 3; *PA* phosphatidic acid, *DAG* 1,2-diacylglycerol, *LPA* Lyso-PA, *PC* phosphatidylcholine, *SM* sphingomyelin, *SMS* sphingomyelin synthase, *ASMase* acidic sphingomyelinase. Enzymes are indicated by *italics*. *Up/down arrows* indicate increase/decrease in expression, activity or levels. ⊣ indicates inhibition

Ceramide can induce cell-cycle arrest through activation of PP1 and PP2A, which dephosphorylate pRb and pCdk2 [22, 25, 63] (Fig. 7). Ceramide also inhibits cell-cycle progression by up-regulating endogenous Cdk inhibitors p21 [25] and p27 [64]. Since D609 was shown to inhibit bFGF-induced astrocyte proliferation, suggesting cell-cycle inhibition, we investigated the effect of D609 on expression of Cdk inhibitors p21 and p27. Expression of p21 was substantially up-regulated in the IC compared with the contra-cortex at 1-day reperfusion following D609 treatment, which corresponded with an increase in ceramide. Ceramide showed a modest (but not significant) increase in the IC without D609 (saline control) at 1-day reperfusion without an increase in p21 expression, suggesting that a threshold level of ceramide may be needed for p21 induction. Expression of p27 showed a much different pattern compared with p21. p27 was expressed in the contra-cortex, whereas p21 was very low or undetectable. This plus the observation that p27 was unaffected by D609 indicates that in rat brain, expression of p21 and p27 are regulated by different mechanisms, although what the regulatory factors are will require further investigation.

The down-regulation of pRb expression in the IC, evidence of cell-cycle arrest, showed a good correlation

with induction of p21, indicating that p21, through inhibition of Cdks, prevented phosphorylation of Rb and is a major factor responsible for decrease in pRb.

In their role as phagocytic cells, microglia/macrophages produce significant amounts of ROS [48, 65, 66], which is primarily produced through the NADPH oxidase system. Upon activation, NADPH oxidase produces the superoxide anion, which leads to formation of other oxidants including $H_2O_2$, hydroxyl radical, and peroxynitrite. In the presence of oxygen radicals, double bonds of unsaturated fatty acids of phospholipids including PC can get oxidized, resulting in an oxidized phospholipid [67]. Scission of the oxidized polyunsaturated fatty acids results in formation of phospholipid aldehydes such as OxPC, and aldehyde cleavage fragments including 4-hydroxynonenal (HNE). These reactive phospholipid aldehydes exhibit cytotoxicity by binding to lysine residues of cellular proteins. OxPC generated under oxidative stress is a marker of inflammation [66] and apoptosis [68, 69]. Recent studies showed that cell-cycle inhibition attenuated microglia proliferation and expression of IL-1ß after tMCAO [70]. Studies have demonstrated that blocking IL-1 signaling provides substantial reduction in infarct after stroke [71]. In addition to preventing neurons from progression into the cell cycle and neuronal death, cell-cycle inhibition by ceramide can attenuate microglial proliferation, expression of IL-1ß, and generation of ROS and OxPC. Alternatively D609 could attenuate ROS/OxPC formation through its antioxidant/glutathione mimetic properties [8, 9].

We have shown previously that D609 inhibited proliferation of cultured neural progenitor cells [72]. The number of BrdU-positive cells was significantly reduced in comparison to controls, indicating that D609 inhibited proliferation of the neurospheres. D609 also inhibited proliferation of primary astrocytes [20] as well as neural stem cells [73]. This could suggest that D609 beneficial effects could arise from inhibiting proliferating microglia/macrophages that are the primary source for TNF-α and IL-1 and other pro-inflammatory cytokines in stroke [74] (see also noted added in proof).

Thus, ceramide can have a neuroprotective effect through induction of p21 and cell-cycle inhibition. p21 can also have a neuroprotective role by inhibition of pro-death signaling through cytosolic interaction with and inhibition of apoptosis signal-regulating kinase 1, thus preventing down-stream activation of stress-activated protein kinase (SAPK/JUN) and induction of cell death [75].

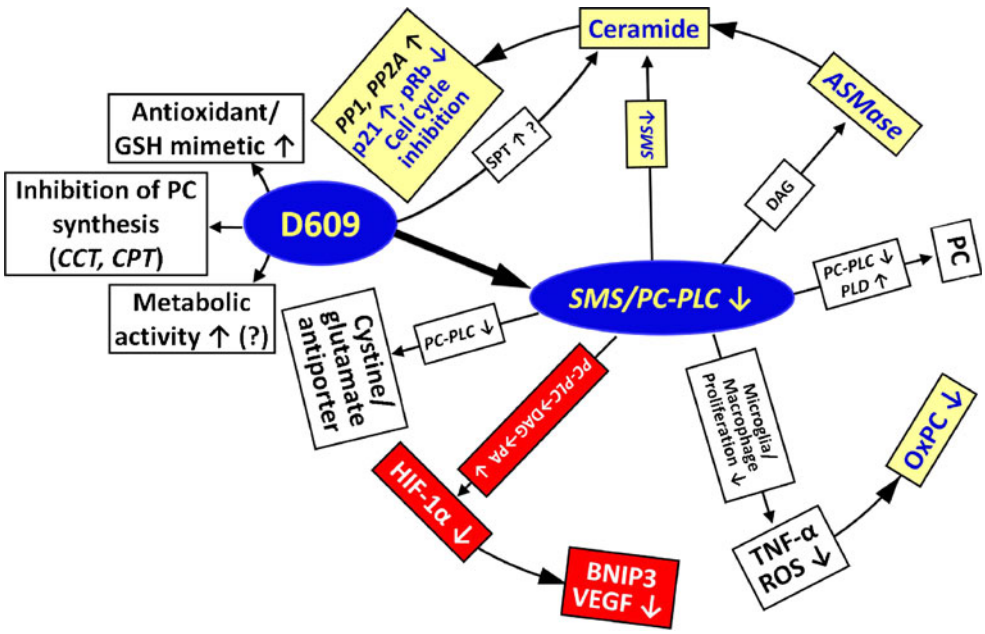


**Fig. 10** Multiple pathways may be affected by D609 through targeting SMS/PC-PLC [6, 7]. There are two forms of SMS (SMS1 in Golgi and SMS2 on the plasma membrane) and D609 inhibits both forms [21]. D609 may increase de novo ceramide synthesis by stimulating SPT [51]. D609 antioxidant/GSH mimetic properties are based on Aß induced oxidative stress [10], effects on cystine/glutamate antiporter are based on glutamate toxicity in immature hippocampal neurons (do not express glutamate receptors) [16], increase in metabolic activity in 264.7 macrophages is based on MTT assay. D609 inhibited PC synthesis and may directly affect membrane translocation of cytidine triphosphate (*CTP*): phosphocholine cytidylyltransferase (*CCT*), the rate-limiting enzyme in PC synthesis [77] or CDP-choline:DAG phosphocholine transferase (*CPT*), the final step in the PC synthesis [78]. D609 prevented 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*)-induced increase in DAG, activation of nuclear CCT activity, and increase in nuclear PC synthesis [79]. Studies indicated that inhibition of PC-PLC by D609-enhanced PLD activity either due to a compensatory effect or that D609 directly increased PLD activity [17]. D609 (750 μM) inhibited $PLA_2$ [19] while 350 μM had no effect [18]. Enzymes are indicated by *italics*. *Up/down arrows* indicate increase/decrease in expression, activity or levels

This action of p21 is independent of its nuclear function as a Cdk inhibitor.

Previous studies attributed activation of HIF-1α to PA formation. PA can be formed from DAG through phosphorylation by DAG kinases. D609 prevented HIF-1α induction (*via* PC → *PC-PLC*→ DAG→ *DAG kinase* → PA→HIF-1α pathway) after tMCAO (these studies used DMSO as a vehicle for D609 when it is soluble in saline) [12]. However these studies did not measure PA formation and did not address the fact that PLD can act directly on PC to generate PA which is major pathway of PA generation (PC→ *PLD*→PA) (Fig. 8). Our previous studies demonstrated activation of PLD after stroke [38].

HIF-1α can induce cell-cycle arrest by up-regulating p21 and p27 [76], and therefore it may be expected that, if HIF-1α is induced after tMCAO, p21 and p27 would be up-regulated, and D609 would attenuate their expression by inhibiting HIF-1α expression. However, in our studies at 1-day reperfusion p21 and p27 were not induced under ischemic conditions and D609 *up-regulated* p21 (Figs. 3 and 4). These observations do not support the role of HIF-1α in p21 induction [12, 32, 76] in our tMCAO model. We attribute p21 induction to ceramide accumulation [22].

In conclusion, our studies as well as those of others [12, 49] show neuroprotection by D609; however the mechanism of action of D609 appears to be different. Here we summarize two proposed actions of D609 in reducing infarction after stroke (a) by decreasing PA and HIF-1α expression and BNIP3 [12] and (b) inhibiting SMS and increasing de novo ceramide synthesis that could increase activity of protein phosphatases PP1 and PP2A, p21 expression and hypophosphorylate Rb (Fig. 9). These differences could be due to severity or duration of ischemia and different species and strains; further studies are necessary to elucidate the mechanisms of D609 actions. Besides these actions, studies in the literature proposed other possible actions of D609, and these are summarized in Fig. 10.

**Acknowledgements** We would like to thank Drs. H. Kalluri, M. Kunnimalaiyaan for helpful discussions, University of Wisconsin Graduate School and School of Medicine and Public Health for supporting this research.

**Note added in proof** Recent studies indicate that ablation of proliferating microglia exacerbates ischemic injury in mice after 1 hr tMCAO and reperfusion [80]. This could also suggest that D609 (one dose given at the onset of reperfusion) may prevent mature neurons from entering the cell cycle at the early reperfusion, however may not interfere with later (2-3 day) proliferation of microglia/macrophages that are the source of trophic factors such as brain derived neurotrophic factor (BDNF) [81] and insulin-like growth factor (IGF-1) in offering protection. Interestingly ablation of proliferating microglia resulted in an increase in the number of cells expressing cytokines TNF-1α, IL-1ß and IL-6. However the identity of these cells was not determined. It will be interesting to resolve whether microglia/macrophages are the primary source of pro-inflammatory cytokines [74] or other cells in the absence of activated microglia/macrophages will still provide the pro-inflammatory cytokines [80]. Defining the role of microglia/macrophages in CNS injury is a challenge since they are the source for both pro-inflammatory as well as neurotrophic factors [81–83].

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