CHAPTER- 5

Mitochondrial dysfunction: A crucial event in Okadaic acid (ICV) induced memory impairment and apoptotic cell death in rat brain

1. Introduction

Mitochondria are unique among cell organelles in their involvement in the concerted consumption of oxygen, production of oxygen radicals, and mobilization of $[\text{Ca}^{2+}]_i$ (Jouaville et al., 1995). The pivotal role of mitochondria in excitotoxicity has been highlighted recently by the deterministic influence of mitochondrial function in the decision between apoptotic and necrotic cell death. Furthermore, Fernandez et al. (1993) has been reported that OKA in hippocampal neuronal cell culture increases $[\text{Ca}^{2+}]_i$ through the ionotropic excitatory amino acid receptors resulting in neuronal degeneration. Tapia et al. (1999) demonstrated that the microinjection of OKA in rat hippocampus induced neuronal stress, hyperexcitation and neurodegeneration. A marked neuronal destruction of the CA1 region and a partial damage of the dentate gyrus were observed following intra hippocampal administration of OKA (Arias et al., 1998). Hippocampus plays the crucial role in memory function and is the most affected brain area in AD (Squire, 1992). Memory deficit induced by OKA has been shown in experimental studies (He et al., 2005).

The pivotal role of mitochondria in excitotoxicity has been highlighted recently in apoptotic and necrotic cell death (Papadia et al., 2008). Apoptosis play a significant role in cell loss during neurodegenerative disorders such as Alzheimer's disease (AD) (Loh et al., 2006). A cascade of events like activation of caspases and aspartate-specific cysteine proteases has been proposed to play a key role in apoptosis (Nicholson et al., 1997). The major apoptotic pathway is characterized by mitochondrial dysfunction with the release of cytochrome c, activation of caspase-9, and subsequently of caspase-3. It has been suggested that caspase-3 is an ultimate effectors caspase whose activation leads to switch on the apoptotic cascade (Lee et al., 2005). Evidences of caspase-3 activation were also found in post-mortem study conducted on the brain of AD patient. (Engidawork et al.,
Biochemical features of apoptosis include loss of plasma membrane phospholipids symmetry and ATP levels (Kroemer et al., 2000). In apoptosis, alteration in mitochondrial activity occurs i.e. activation of cysteine proteases of caspase family; mitochondrial membrane depolarization, mitochondrial oxyradical production and calcium overload (Keller et al., 1998). The mitochondria are thought to be both generators of reactive oxygen species (ROS) and targets of ROS attack. Indeed, ROS generation is an important mechanism accounting for cellular injury in many neurodegenerative disorders (Valko et al., 2006). The glutamatergic synapses are most likely site for initiation of neurodegenerative process. Activation of glutamate receptors is believed to play a major role in the neuronal cell death (Mattson et al., 1996). Activation of glutamate receptors causes massive calcium influx through N-methyl-D-aspartate receptors, voltage-dependent calcium channels (Choi et al., 1994) and oxyradical production (Mattson et al., 1996).

2. Methods

The crude mitochondrial fraction was isolated according to Weiler et al, (1981). The biochemical parameter related to mitochondrial function and apoptotic cell death was estimated in different brain areas. Mitochondrial Calcium ion was estimated fluorometrically followed by Grynkiewicz et al, (1985). ATP was estimated in rat brain using ATP colorimetric assay kit (Biovision). Caspase-3 and 9 activities were estimated fluorometrically and expressed in μmoles /mg of protein. The mRNA expression Caspase-3 and 9 were done by RT PCR method. Membrane potential was analyzed by using Rhodamine123(R-123) and SDH activity was estimated spectrophotometrically at 570 nm according to method followed by Castoldi et al, (2000).
3. Results

3.1. Mitochondrial function: A studies in mitochondrial preparation

3.1.1. Reactive oxygen species (ROS)

Production of reactive oxygen species (ROS) in brain regions was measured relative to control. There was a significant increase (P<0.01) in ROS level in cerebellum, hippocampus, cortex and striatum of OKA 200 ng treated rats as compared to control group. Treatment with memantine significantly (P<0.05) reduced amount of ROS whereas donepezil did not show significant (P>0.05) effect in any brain regions (Fig.7).

![Graph showing ROS levels in different brain regions with significance markers](image)

**Fig.7.** Figure represents that only memantine was able to reduced ROS level in OKA induced ROS generation where as donepezil was failed to restore the OKA induced ROS generation in rat. *P< 0.01, **P< 0.001 vs. control group and *P< 0.01, **P< 0.001 vs. OKA group.
3.1.2. Mitochondrial calcium ion $\text{Ca}^{2+}$

There was a significant ($P<0.01$) increased in $\text{Ca}^{2+}$ in hippocampus, cortex, striatum and cerebellum of OKA 200 ng treated rats as compared to control and aCSF treated rat. Treatment with memantine and donepezil significantly ($P<0.01$) reduced amount of $\text{Ca}^{2+}$ in OKA treated rat brain regions (Fig. 8).

Fig. 8. Okadaic acid significantly increases $\text{Ca}^{2+}$ level as compared to control and aCSF group. Treatment with memantine and donepezil significantly reduced $\text{Ca}^{2+}$ level in OKA injected rats. *$P<0.01$, **$P<0.001$ control group and *$P<0.01$, **$P<0.001$ vs. OKA group.
3.1.3. Mitochondrial membrane potential (MMP)

There was a significant (P<0.05) decreased in MMP in hippocampus and cortex of OKA 200ng treated rats as compared to control and aCSF treated rat. Treatment with memantine significantly (P<0.05) increased MMP in cortex and hippocampus, where as donepezil significantly (P<0.05) increase MMP in cortex, hippocampus and striatum as compared to OKA 200ng treated rat (Fig.9).

![Graph showing MMP levels in different areas](image)

**Fig.9.** A significant decreased in MMP level in mitochondrial preparation was observed in cortex and hippocampus of OKA (200 ng) treated rats which was normalized by memantine and donepezil. *P* < 0.01 vs. Control group and *P* < 0.05 vs. OKA group.
3.2. Studies in synaptosomal preparation

3.2.1. ATP level

ATP content was estimated by ATP assay kit (Biovision) in rat brain to assess Energy metabolism. OKA administration significantly (P<0.01) decreased ATP content in rat brain areas hippocampus, cortex and striatum as compared to control and aCSF group. ATP level in hippocampus, striatum, cerebellum and cortex was significantly increased (P<0.01) following treatment with memantine, whereas treatment with donepezil significantly (P<0.01) increased ATP level in hippocampus, cortex and striatum as compared to OKA 200ng treated rat (Fig. 10).

![Graph showing ATP level comparison](image)

**Fig.10.** Figure represents that OKA reduced ATP level in cortex, hippocampus and striatum whereas donepezil and memantine was able to restore the Okadaic acid induced ATP depletion in rat. *P<0.01, **P<0.001 vs. control group and *P<0.01, **P<0.001 vs. OKA group.
3.2.3. SDH activity

There was significant (P<0.05) less SDH activity observed in hippocampus and cortex of OKA 200ng treated rats as compared to control and aCSF treated rats. Preventive treatment daily for 13 days with memantine and donepezil restored mitochondrial activity in cortex and hippocampus as shown by significant (P<0.05) increased in SDH activity (Fig.11).

Fig.11. Shows that OKA (200ng) reduced mitochondrial activity in cortex and hippocampus in rat brain, which was reversed by donepezil and memantine in rat. *P<0.01 vs. control group and *P<0.01 vs. OKA group.
3.2.4. Malondialdehyde (MDA) level

OKA (200ng) increased significantly (P<0.01) MDA level in hippocampus, cerebellum, striatum and cortex as compared to control and aCSF group. Pretreatment with memantine significantly (P<0.01) decreased MDA level in hippocampus, cerebellum, whereas treatment with donepezil significantly decreased (P<0.001) MDA in hippocampus, cerebellum, cortex, and striatum as compared to OKA 200ng treated rat (Fig.12).

![Graph showing MDA levels in different brain regions](image)

**Fig.12.** A significant increase in MDA level was observed in case of OKA treated rat as compared to control and aCSF treated rats. Both memantine and donepezil significantly reduced MDA level in OKA treated rats. ##P< 0.001 vs. control group and *P< 0.05, **P< 0.005, ***P< 0.001 vs. OKA group.
3.2.5. Total Nitrite estimation

Nitrite level in the brain regions was measured at the end of the experiment. As shown in fig. 13, nitrite levels were significantly (P<0.001) elevated in cortex and hippocampus of OKA 200ng treated rat brain. Both memantine and donepezil significantly prevented (P<0.001) this increase in nitrite levels in different brain areas of OKA 200ng treated rat.

**Fig.13.** A significant increased in nitrite level was observed in cortex and hippocampus of OKA (200ng) treated group, which was reversed by memantine and donepezil. *P<0.01 vs. Control group and *P<0.05, **P< 0.001 vs. OKA group.
3.2.6. Caspase-3 activity

A significant (P<0.01) increased in caspase-3 activity was observed in hippocampus, striatum and cortex of OKA treated rat brain in comparison to that of control and aCSF group. Treatment with memantine and donepezil significantly (P<0.05) decreased caspase-3 activity and mRNA level in hippocampus, striatum and cortex of OKA 200ng treated rat brain (Fig. 14).

**Fig.14.** A significant increased in caspase-3 in activity was observed in striatum, cortex and hippocampus of OKA treated group which was normalized by memantine and donepezil. #P< 0.01 vs. Control group *P< 0.05 and **P< 0.001 vs. OKA group.
3.2.7. Caspase-9 activity

A significant (P<0.01) increase in activity caspase-9 was observed in hippocampus and cortex of OKA treated rats as compared to that of control and aCSF group. Treatment with memantine and donepezil significantly (P<0.01) decreased caspase-9 activity and mRNA expression in hippocampus and cortex as compared to OKA 200ng treated group (Fig.15).

![Caspase-9 activity graph](image)

**Fig.15.** A significant increased in caspae-9 activity was observed in cortex and hippocampus of OKA treated group which was reversed by memantine and donepezil. #P< 0.01 vs. Control group, **P< 0.005 and *P< 0.05 vs. OKA group.

3.2.8. Caspase-3 mRNA expression

A significant (P<0.01) increased mRNA expression of caspase-3 was observed in hippocampus, striatum and cortex of OKA treated rat brain in comparison to that of control and aCSF group. Treatment with memantine and donepezil daily for 13 days significantly (P<0.05) decrease caspase-3 mRNA level in hippocampus, striatum and cortex of OKA 200ng treated rat brain (Fig.16).
Fig. 16. A significant increase in caspase-3 mRNA was observed in striatum, cortex and hippocampus of OKA treated group, which was reversed by memantine and donepezil.

#P < 0.01 vs. Control group and *P < 0.05 vs. OKA group.
3.2.9. Caspase-9 mRNA expression

A significant (P<0.01) increase in mRNA expression of caspase-9 was observed in hippocampus and cortex of OKA treated rats as compared to that of control and aCSF group. Treatment with memantine and donepezil significantly (P<0.01) decreases caspase-9 mRNA expression in hippocampus and cortex as compared to OKA 200ng treated group (Fig.17).

![Diagram showing mRNA expression levels in different brain regions and treatments.](image)

- **Control**
- **OKA (200ng)**
- **OKA + (Don 5mg/kg)**
- **aCSF**
- **OKA+ (Mem10mg/kg)**

Cerebellum, Striatum, Cortex, Hippocampus

M- Molecular weight marker, 1- control, 2- aCSF, 3- OKA, 4- Memantine and 5- Donepezil
Fig.17. Okadaic acid (200ng) induces caspase-9 mRNA up regulation in cortex and hippocampus whereas pretreatment with memantine and donepezil reversed the same. 

*P< 0.01 vs. Control group **P< 0.005 vs. OKA group.

4. Discussion:

Effectiveness of donepezil and memantine indicates the involvement of cholinergic and NMDA mechanisms in OKA induced memory impairment. Glutamate neurotoxicity is triggered primarily by massive [Ca2+]i influx arising from overstimulation of the NMDA subtype of glutamate receptors. Excessive [Ca2+]I accumulation in mitochondria uncouples electron transfer from ATP synthesis (Bernardi et al., 1994) and impairment of energy metabolism increases generation of free radicals (Wang et al., 1994). Thus, mitochondria emerges as a plausible link between elevation of [Ca2+]i and glutamate neurotoxicity. Therefore, cholinergic and mitochondrial activity were investigated in OKA induced memory impaired rats at biochemical and cellular level. Further, the studies were carried out to explore the status of mitochondrial function and apoptotic cell death in brain areas related with memory functions in OKA treated rats.

Mitochondrial dysfunction is considered to play a cardinal role in the pathogenesis of various neurodegenerative disorders like AD. [Ca2+]i ion plays a key role as a regulator of numerous cellular functions. It is proposed that rise in levels of intracellular [Ca2+]i through NMDA receptor and voltage-gated calcium channels leads to the impairment in the mitochondrial electron transport system which consequently result into generation of intracellular nitric oxide radicals and ROS leading to tissue damage (Bonfoco et al., 1995). The studies have shown that ROS and [Ca2+]i regulation is tightly bound and that disruption in either could affect the other. Elevated ROS level causes an imbalance in [Ca2+]i regulation in mitochondria due to compromised cellular lipid bilayer (Berridge et al., 2000).

Effectiveness of memantine indicates that OKA might be causing excitotoxicity through increase in [Ca2+]i that causes neurotoxicity. In the present study, we found significant elevation in mitochondrial [Ca2+]i in OKA treated rat compare to control and aCSF treated rats. Therefore, [Ca2+]i and ROS level in mitochondrial are considered as an
indicator of mitochondrial function (Mungarro et al., 2002). Treatment with both anti-dementic drugs memantine and donepezil showed significant reduction in [Ca2+]i level in OKA treated rats. This observation indicates that OKA might be acting through glutaminergic system as NMDA antagonist memantine and anticholinergic donepezil blocked OKA induce elevation of mitochondrial [Ca2+]i. Donepezil is reported to exert protective effect against intracellular [Ca2+]i and glutamate toxicity in cultured cortical neurons (Akasofu et al., 2006). Thus it appears that neuronal dysfunction particularly in hippocampus due to free radicals and mitochondrial [Ca2+]i may be the principal factors for OKA induced memory impairment.

Mitochondrial membrane lipids especially the long chain polyunsaturated fatty acid components are highly susceptible to ROS. Lipid peroxidation could cause structural damage to mitochondrial membranes and potentiate their dysfunction. Recently, it has been observed that lipid peroxidation in the brain occurs in early AD (Williams et al., 2006). Therefore, an elevated lipid peroxidation may indicate neuronal degeneration. The rise in level of MDA, a product of lipid peroxidation, following OKA administration in synaptosomal preparation of cortex, hippocampus and striatum of rat brain may be linked with neuronal damage. We also observed a significant rise in nitrite levels in brain of OKA treated rat. The concomitant rise in ROS and nitrite may leads to the formation of peroxynitrite, a powerful pro-oxidant (Cosentino et al., 1997), NO and its toxic metabolite, peroxynitrite can inhibit components of the mitochondrial respiratory chain leading to a cellular energy deficiency state ultimately activating cell death pathways (Cassina et al., 1996). Further, excessive Ca2+ accumulation in mitochondria along with increased free radical formation causes defects in electron transport chain resulting into impaired mitochondrial function due to membrane depolarization and decreased ATP production (Tota et al., 2011). The enzyme succinate dehydrogenase (SDH) is responsible for reduction of MTT, in the mitochondria of rats (Pollak and Duck-Chong, 1973). In the present study OKA also reduced mitochondrial metabolic function as indicated by less SDH activity in the MTT assay. In this study, OKA also caused a significantly decreased in ATP production, MMP and mitochondrial metabolic activity in cortex, hippocampus and striatum brain regions except cerebellum indicating impairment in energy metabolism and mitochondrial function. This may be due to alteration in
mitochondrial Ca$^{2+}$ and free radical formation by OKA. A decrease in mitochondrial membrane potential (MMP) is an early universal event of apoptosis (Wadia et al., 1998). The release of mitochondrial cytochrome C and apoptosis-inducing factor are key events in initiating the cascade of reactions leading to apoptotic cell death (Nicholls et al., 2000). It was reported that structural and functional changes in mitochondria play a central role in apoptosis (Desagher et al., 2000).

The extrinsic pathway is activated at the cell surface through death receptor mediated activation of caspase-8 or caspase-10, followed by caspase-3 activation and this pathway may be amplified by caspase-9 activation i.e. intrinsic pathway (Selkoe et al., 2002). Therefore, caspase-3 and caspase-9 are main initiator caspses which plays a pivotal role in the progression of a variety of neurological disorders. Despite the various causes of such disorders, the mechanism of cell death is similar in a broad spectrum of neurological diseases (Yuan et al., 2000). However, the trigger of aberrant caspase activation in majority of these diseases is not well understood. In acute neurological diseases, both necrosis and caspase-mediated apoptotic cell death occur (Emery et al., 1998). By contrast, in chronic neurodegenerative diseases, caspase-mediated apoptotic pathways have the dominant role in mediating cell dysfunction and cell death (Li et al., 2000).

In this study, we have investigated the effect of OKA on caspase-3 and caspase-9 level in rat brain. OKA significantly increased activity and mRNA expression of caspase-3 and caspase-9 in rat brain areas indicating involvement of caspase mediated cell death in OKA induced neurodegeneration. Mitochondrial dysfunction induced by OKA could be an important function for initiation of apoptosis.