6. TNF-α regulates oxidative stress, mitochondrial function and autophagy in neuronal cells

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6.1 TNF-α induces mitochondrial oxidative stress in SH-SY5Y cells.

The dysregulation of mitochondria and oxidative stress is one of the primary features of PD stress conditions. The activation of caspase-9 and caspase-3 cascade suggests the dysregulation of mitochondrial functions in the presence of TNF-α, which may be one of the reasons for increased cell death in PD stress conditions. To elucidate the role of TNF-α in oxidative stress, the generation of ROS at mitochondria was monitored by MitoSOX staining. The dye permeates live cells where it is selectively targeted to mitochondria, oxidized by superoxide and generates red fluorescence. The number of MitoSOX positive cell significantly increased in the presence of TNF-α as compared to untreated cells (Fig 6.1A) suggesting the induction of mitochondrial ROS. Quantification of MitoSOX fluorescence was performed by spectrofluorometry method using rotenone as positive control. The significant increase in fluorescence intensity was observed in presence of TNF-α (Fig 6.1B) suggesting the generation of mitochondrial ROS. The generation of ROS may lead to lose of mitochondrial membrane potential (ΔΨ), hence TMRM staining was performed in presence and absence of TNF-α. The fluorescence intensity significantly decreased in the presence of TNF-α (Fig 6.1C). To further confirm the role of TNF-α induced oxidative stress in induction of DAergic neuronal death, the cell survival was monitored in the presence of antioxidant (NAC) and caspase inhibitor (z-VED.fmk). TNF-α induced cell death was rescued by NAC and z-VED.fmk (Fig 6.1D). The activity of caspase3/7 significantly increased in the presence of TNF-α and decreased in the presence of antioxidant and caspase inhibitor (Fig 6.1E). The results here demonstrated that TNF-α induces oxidative stress and subsequently activates caspase dependent cell death pathway in SH-SY5Y cells.

Figure 6.1

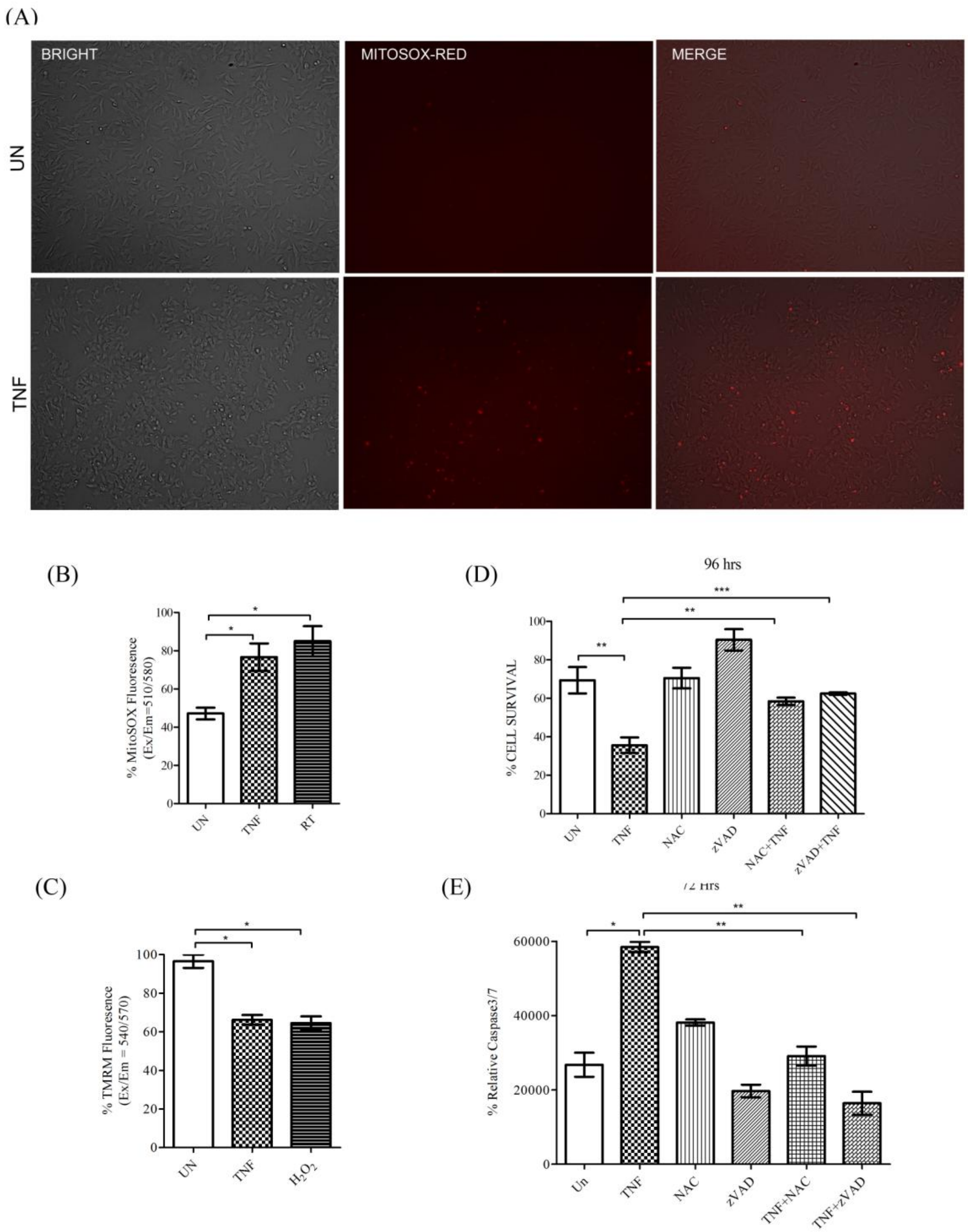


Figure 6.1: TNF-α induces mitochondrial oxidative stress and cell death.

(A) SH-SYSY cells were treated with TNF-α (20 ng/ml) for 48 hours. MitoSOX staining

was performed to monitor ROS production using fluorescence microscope. (B) SH-SY5Y cells were treated with TNF-α (20 ng/ml) for 48 Hrs. Quantitative assay for mitochondrial ROS was performed using Mito-SOX by spectrofluorometry. Rotenone treated cells were used as positive control for 4 hours. (C) SH-SY5Y cells were treated with TNF-α (20 ng/ml) for 48 Hrs. Change in mitochondrial membrane potential was performed using TMRM by spectroflurometry. H_2O_2 treated cells were used as positive control for 2 Hrs. (D and E) SH-SY5Y cells were treated with TNF, NAC and z-VAD.fmk either alone or in combination. (D) After 96 hours of treatment, cell survival was assessed by MTT assay and (E) caspase 3/7 activity was measured using caspases 3/7 Luciferase assay. Asterisk (*) indicates that p value <0.05, for SEM

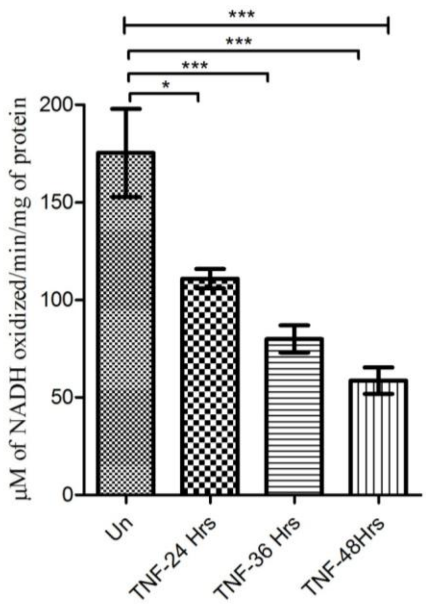
6.2 TNF-α regulates mitochondrial complex-I in dopaminergic cells.

The above experiment showed increased oxidative stress in the presence of TNF-α suggesting compromised mitochondrial functions. The mitochondrial complex-I is the entry point of the electrons in the electron transport chain (ETC) (Sterky and Larsson, 2008). The dysregulation of mitochondrial complex-I lead to leakage of electrons and is one of the primary sources of ROS. However, its regulation in the presence of TNF-α and its implication in PD stress is not well understood. Hence, we monitored the mitochondrial complex-I activity by spectrophotometric assay and BN-PAGE. The spectrophotometric assay showed that complex-I activity decreased significantly in the presence of TNF-α. The decrease activity was observed as early as 36 hours where as the caspase activation and cell death was evident after 72 hours of TNF-α treatment as compared to untreated cells (Fig 6.2A). To further confirm this observation, BN-PAGE was performed and in-gel assay for complex-I showed decreased activity in the presence of TNF-α treated cells as compared to untreated cells (Fig 6.2B). This suggests that dysregulation of complex-I precede the cell death in the presence of TNF-α.

TNF-α mediated decrease in mitochondrial complex-I activity may also affect ATP levels in the cells. The total cellular and mitochondrial ATP levels were determined in the presence/absence of TNF-α. In TNF-α treated cells, both total cellular and mitochondrial ATP levels were significantly decreased as compared to untreated cells (Fig 6.2C and D). These evidence strongly suggest that mitochondrial functions may be dysregulated in the presence of TNF-α.

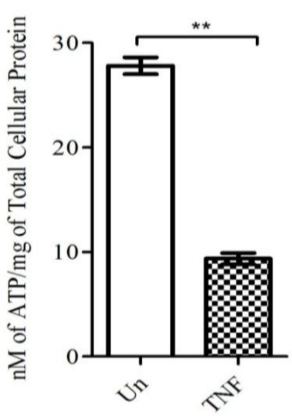
Figure 6.2

(A)



(B)

(C)



(D)

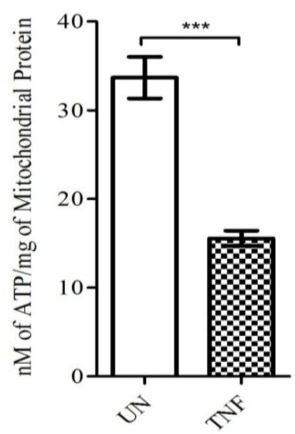


Figure 6.2: TNF-α regulates mitochondrial complex-I activity in SH-SY5Y cells.
(A) SH-SY5Y cells were treated with TNF-α at different time period. Complex-I activity was measured at indicated time point of TNF-α treatment by monitoring the decrease in absorbance at 340 nm. (B) Blue Native gel electrophoresis of Triton-X 100 solubilized

mitochondrial extracts from TNF-α treated and untreated SH-SY5Y cells were performed. The gel strips were stained with complex-I staining solution. (C and D) After 72 hours of TNF-α treatment, ATP levels in untreated and TNF-α treated condition were measured by ATP-dependent luciferase activity. (C) ATP level in whole cell lysates (D) ATP level in mitochondrial fraction. Asterisk (*) indicates that p value <0.05, for SEM.

6.3 TNF-α modulates mitophagy.

The mitochondrial homeostasis is required for the optimal function of the cell (Kulkarni *et al.*, 2007). The defective mitochondria are eliminated by selective process of autophagy called as mitophagy (Hattori *et al.*, 2014; Redmann *et al.*, 2014). However, TNF-α mediated regulation of mitophagy and its implication in PD disease is not well understood. The level of autophagy was also analyzed in the presence of TNF-α by fluorescence microscopy and western blotting of LC3. The number of LC3-GFP puncta formation was increased in presence of TNF-α and NH_4CL as compare to untreated (Fig 6.3.1A & C). To further confirm the effect of TNF-α on flux, we analyzed the turnover of LC3 using tandem mCherry-GFP constructs. The tandem mCherry-GFP is an interesting tool to monitor autophagy flux (Mizushima *et al.*, 2010). It is known that GFP has higher pKa value (pKa = 6.2) compared to mRFP (pKa = 4.8) suggesting that mRFP is resistant to lysosomal degradation and does not lose its fluorescence when autophagosome fuses with lysosome whereas GFP loses its fluorescence within lysosome. Therefore autophagosome are observed as orange color puncta whereas autophagolysosomes as red colored puncta, as described previously (Mizushima *et al.*, 2010). To determine autophagy flux, SH-SY5Y cells were transfected with mCherry-GFP-LC3, puncta formation was monitored in the presence of TNF-α. Interestingly, the number of red puncta increased significantly in TNF-α treated cell as compared to untreated cells (Fig 6.3.1B & D). The levels of lapidated form of LC3-II increased in the presence of TNF-α and its turnover increased till 36 hours as the levels of LC3-II form increased in the presence of bafilomycin. Interestingly, the level of LC3-II did not change in the presence of bafilomycin (vacuolar H+ ATPase (V-ATPase)) after prolonged

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Figure 6.3.1

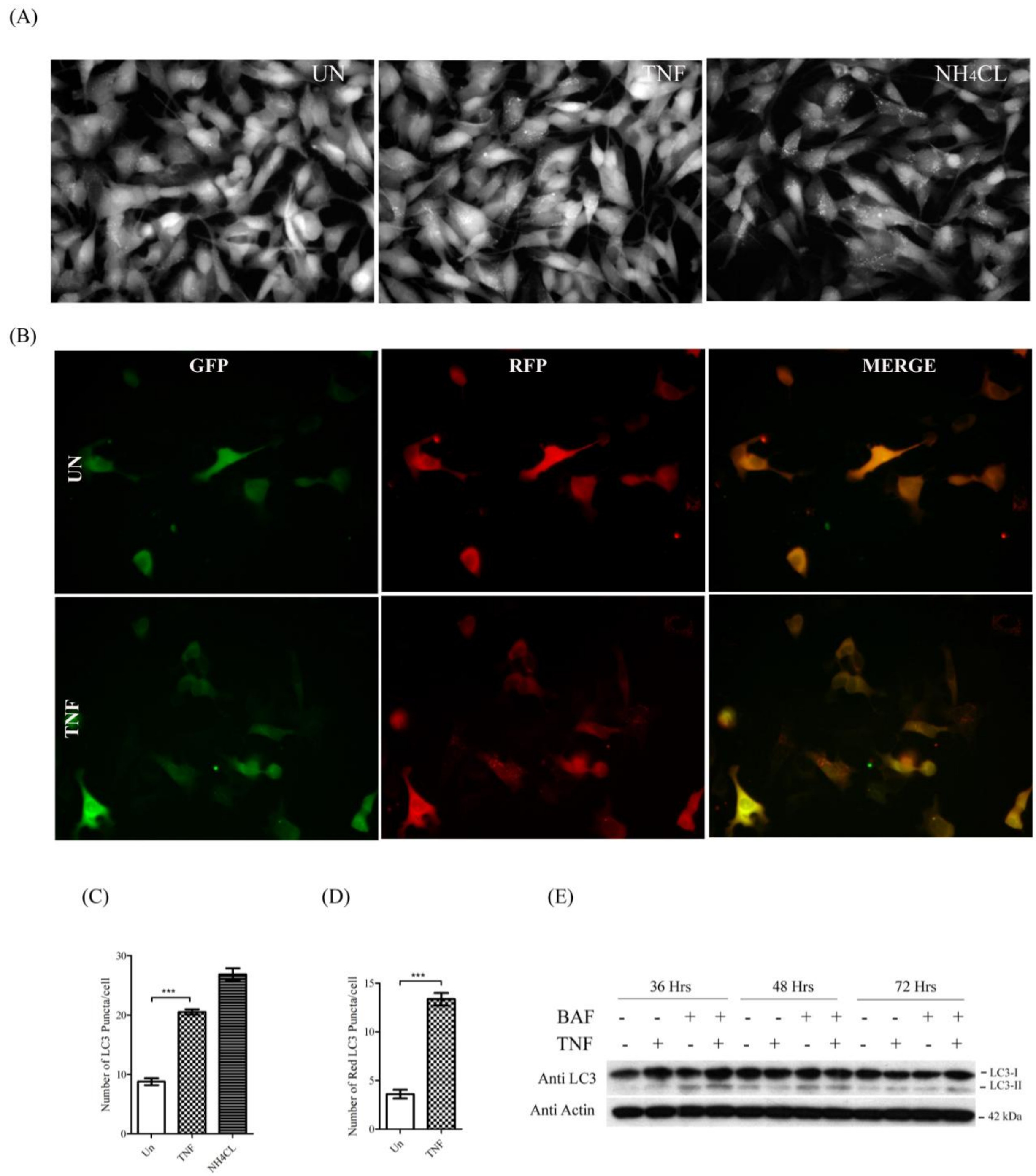

Figure 6.3.1: TNF-α modulates autophagy.

(A) SH-SY5Y-LC3-GFP stable cells were treated with TNF-α for 72 hours and NH_4CL for 4 hours and GFP-LC3 punct formation was monitored using fluorescence microscopy.

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(B) SH-SY5Y cells were transfected with mCherry-GFP-LC3. Cells were treated with TNF-α for 72 hours and autophagy flux was monitored under fluorescence microscopy for LC3 red puncta. (C) Graphical representation of GFP-LC3 puncta. Numbers of LC3-GFP puncta were counted for minimum 20 cells and average numbers of puncta per cell were plotted for Untreated and TNF-α conditions. (D) Numbers of LC3 red puncta were counted for minimum 20 cells and average numbers of puncta per cell were plotted for Untreated and TNF-α condition. (E) SH-SY5Y cells were treated with TNF-α at indicated time point and co-treated with bafilomycin (lysosomal inhibitor) for 10 hrs. The LC3-I and LC3-II forms were monitored by western blotting using LC3 antibodies. Asterisk (*) indicates that p value <0.05, for SEM

Figure 6.3.2

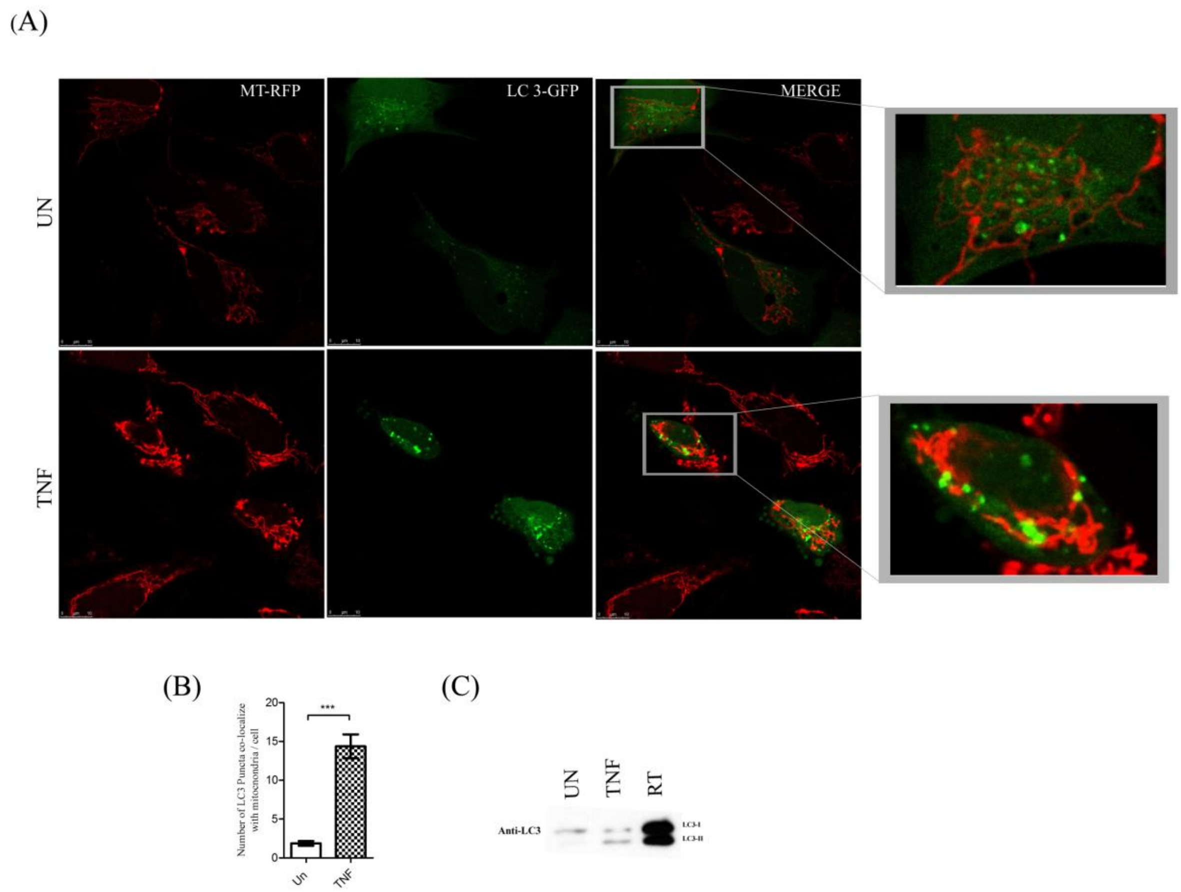

Figure 6.3.2: TNF-α modulates mitophagy.

(A) SH-SY5Y-MTRFP stable cells were transfected with GFP-LC3. Cells were treated with TNF-α for 72 hours and co-localization of GFP-LC3 with red mitochondria was analyzed by confocal microscopy. Scale bars, 10 µm. (B) Graphical representation of the

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numbers LC3 puncta co-localized with mitochondria per cell in untreated and TNF-α treated condition of representative. (C) SH-SY5Y cells were treated with TNF-α for 72 hours and mitochondrial fraction was prepared using differential centrifugation method and association of LC3 with mitochondria was analyzed by western blotting using LC3 specific antibody.

incubation of 48 and 72 hours, which inhibits the lysosomal acidification (Yoshimori *et al.*, 1991), suggesting the decreased turnover of autophagosome to the lysosome (Fig 6.3.1E).

Mitophagy was monitored by co-localization of autophagy marker (LC3 protein) with mitochondria by confocal microscopy and western blotting. SH-SY5Y-MTRFP stable cell line was transfected with GFP-LC3 and co-localization was analyzed by confocal microscopy. The co-localization of green LC3 puncta and red mitochondria was clearly observed in the presence of TNF-α as compare to untreated cells (Fig 6.3.2A) and significantly increased in the presence of TNF-α (Fig 6.3.2B). We also hypothesized that mitochondria undergoing mitophagy may be conjugated with LC3 hence we performed western blotting in the presence/absence of TNF-α and rotenone (as positive control for mitophagy inducer). The LC3-II forms, which represents the lipidated form of LC3, increased significantly in the mitochondrial fraction in presence of rotenone (inhibitor of complex-I, positive control). The lipidated form of LC3 increased in the presence of TNF-α as compared to untreated cells however it was low as compared to rotenone (Fig 6.3.2C). Taken together, all these suggest the increased levels of TNF-α alters the mitochondrial complex-I activity, induces oxidative stress, and alter mitochondrial turnover through mitophagy which may have important implication in PD conditions.

6.4 Discussion:

Mitochondrial dysfunction is one of the cardinal features of the PD (Gautier *et al.*, 2014). TNF-α is known to regulate mitochondrial functions by enhancing ROS, superoxide and H_2O_2 production (Mariappan *et al.*, 2009) . These functions are critically regulated by mitochondrial complex-I which is the entry point of the electrons in ETC. Hence, we hypothesized that the prolonged exposure of TNF-α may decrease complex-I activity and mitochondrial ATP production. The prolonged exposure of SH-SY5Y cells to TNF-α showed decrease in mitochondrial complex-I and ATP levels and induces ROS and cell death. The cell death induced by TNF-α was rescued by antioxidants that further emphasized the role of ROS and complex-I. The evidence of the regulation of mitochondrial complex-I activity by TNF-α suggests its important implication in PD stress conditions. The hypothesis is further supported by observation in animal model of ventricular dysfunction where TNF-α regulate ROS production by down regulating the expression of four subunits of the complex-I (Mariappan *et al.*, 2012). These observations should be validated in animal models and post-mortem brain samples obtained from PD patients.

The defective mitochondria are ubiquitnated through PARKIN and degraded through selective process of autophagy called as mitophagy (Narendra *et al.*, 2008). Mitochondrial homeostasis is critical for neuronal functions hence any alteration in mitochondrial biogenesis or the degradation through mitophagy may lead to neurodegeneration (Chen and Chan, 2009). The evidences here strongly suggest that the turnover of mitochondria is regulated by TNF-α. Mitochondria are recognized by the autophagic machinery and conjugated by LC3 in presence of TNF-α. The induction of mitophagy is in the presence of rotenone in consonance with earlier reports (Frank *et al.*, 2012; Chu *et al.*, 2013). The LC3 conjugation to mitochondria is less prominent in the presence of TNF-α as compare to rotenone. This suggests that defective mitochondria may be accumulated as observed by decreased turnover in the presence of TNF-α leading to generation of ROS and cell death. The levels of mitophagy and its association with TNF-α should be also studied in the clinical samples of PD patient.

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