Bax modulates neuronal survival while p53 is unaltered after Cytochrome C induced oxidative stress in the adult olfactory bulb in vivo

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ABSTRACT

Background: The granule and periglomerular cells of the olfactory bulb migrate from the subventricular zone (SVZ) as progenitor cell forming the neuronal stream of the rostral olfactory bulb. These cells are characterized by their ability to divide while expressing adult proteins; a phenomenon attributed to the prolonged cell cycle and the regulatory activities of proteins which modulates apoptosis and proliferation in the developing nervous system. Of interest are the proteins concerned with tumor suppression (p53) and cell cycle exit (Bax) and how they regulate survival of these neurons in the adult system after an induced oxidative stress.

Purpose: This study sets to investigate the interplay between p53 and Bax in the adult olfactory bulb (periglomerular and granule cell layer), and how these proteins determine proliferation and neuronal survival after Cytochrome C induced-oxidative stress. Also, we demonstrate the effect of the induced-stress threshold on such regulation in vivo.

Methods: Adult Wistar rats were segregated into three groups. 10 and 20 mg/Kg BW of potassium cyanide (KCN) was administered to the treatment groups for 15 days while the control received normal saline for the same duration. The olfactory bulb was dissected and processed for general histology and immunohistochemistry of p53/Bax in the periglomerular and granule cell layers. Total protein and immunopositivity of (p53 and Bax) cell count was done using Image J. Subsequently, we determined the analysis of variance with significance set at *P<0.05.

Results: We observed an increase in cell count for the 10 mg/KgBW treatment; this was characterized by a significant decrease in Bax expression and no change in p53 expression when this treatment group was compared to the control. However, no change was observed in the total cell count for 20 mg/Kg BW treatment for the same duration of exposure. Interestingly, there was also no significant change in Bax and p53 for this treatment when compared with the control.

Conclusion: Although p53 plays an important role in development of the olfactory bulb neurons, our findings suggest it has little contribution in neuronal cell viability and proliferation in the adult olfactory bulb. No significant change in p53 was observed irrespective of treatment dose and cell count while Bax expression was reduced at 10 mg/Kg BW treatment and was associated with an increased cell count. We conclude that regulation of survival of neurons in the adult olfactory bulb, following induced-oxidative stress was more dependent on the expression of Bax and the threshold of the induced stress rather than p53 expression.

Chemical agents (such as cyanide and other heavy metals) inhibit the oxygen carrying capacity of the Cytochrome C (Heme a3-CuI) binuclear center leading to generation of ROS in the mitochondria matrix (oxidative stress). The product of this stress pattern is the release of Cytochrome C (Cyto C) into the cytoplasm which fixes Bax into the membrane of the mitochondria through tBid to create calcium surge in the cytoplasm. Factors which facilitate the up regulation of Bax are usually associated with senescence, reduction of Bcl-2 and over expression of p53. However, other non-Bax related mechanisms are known to induce increased expression of p53 in neurons during oxidative stress. Nitric oxide (NO), formed through the reaction of ROS with nitrogen containing compounds trigger apoptosis by increasing the expression of p53 in the nucleus and subsequently DNA cleavage. These mechanisms are also important in tumor protection and self-renewal in the olfactory bulb through regulation of senescence, tumorgenesis and apoptosis.

The relationship between Bax and p53 expression in the cell cycle of granule and periglomerular cells of the adult olfactory bulb is still relatively unknown. Furthermore the functional role of p53 in the prolonged cell cycle, oxidative stress, neuronal survival and apoptosis in the adult olfactory bulb is yet to be elucidated. Previous studies have demonstrated dose-dependent mechanisms in oxidative stress cytotoxic response in different parts of the brain (also dependent on the...
cell cycle specific for each cell type. A common evidence for the role of the cytotoxic pathways is how the plastic brain regions respond differently when compared with the non-plastic cortex to the same assault. Also, neurons in these regions follow varying patterns of cell death for the same assault and threshold of assault. The role of cell cycle specific for each cell type in regulating proliferation and apoptosis in oxidative stress is a major switch that determines selective vulnerability of adult neurons in the olfactory bulb to varying thresholds of induced oxidative stress. In this study, we have elucidated the interplay of \( p53 \) and \( Bax \) expression in peri-gglomerular and granule cells of the olfactory bulb. We investigated pharmacologically, whether the induction of oxidative stress through inhibition of CcOX at varying doses will generate a difference in \( p53 \)/\( Bax \) expression in the neuroplastic olfactory bulb. Furthermore, we sought to identify the relationship between \( p53 \)/\( Bax \) expression and cell viability in the adult olfactory bulb following induced oxidative stress.

**Methods**

**Treatment**

\( N = 15 \) adult Wistar rats (males; weighing 250–280 gms) were divided into three groups of \( n = 5 \) animals each. Potassium cyanide (KCN) salt was dissolved in PBS (freshly prepared) and was administered orally using a gavage to \( n = 5 \) animals at 20 mg/Kg body weight (BW). Using the same treatment method, a separate set of \( n = 5 \) animals received 10 mg/kg BW of KCN to block mitochondria CcOX (Heme a3-Cu binuclear center) and facilitate cellular release of Cytochrome C and ROS. The control group (\( n = 5 \)) were treated with normal saline. The total treatment duration was 15 days for all groups. Author completed the IACUC training on Animal Use and a protocol number was already assigned to group. (ABU13/Neu/008).

**Olfactory Bulb Tissue Preparation**

Animals were anaesthetized using sodium pentobarbital (45 I.P) and decapitated to obtain the olfactory bulb. All protocols were approved by the Animal Use Ethics Committee of the Aba Babalola University. The brains were quickly submerged into cold artificial cerebrospinal fluid [ACSF: 125 mM NaCl, 25 mM NaHCO\(_3\), 3 mM KCl, 1.25 mM NaHPO\(_4\), 1 mM MgCl\(_2\), 2 mM CaCl\(_2\); fresh ACSF, p53 and \( Bax \) immunohistochemistry to determine the overall cell count and protein specific expressions in these layers. In general histology (Figure 1A) the cell count for 10 mg/Kg treatment increased when compared to the control (\( ***P<0.001 \)) while no significant change in cell count was seen for the 20 mg/Kg treatment versus the control (Figure 1B). However, the increase in cell number in the 10 mg/kg treatment was not associated with any change in \( p53 \) expression in the olfactory bulb (Figure 2A).

This is intriguing as the \( p53 \) expression level in the control and 20 mg/Kg also showed no significant change (Figure 2A and 2B) similar to the observations in the total cell count (Figure 1A and 1B). Thus, for varying thresholds of induced oxidative stress, no change in \( p53 \) expression despite a change in cell count at 10 mg/kg BW treatment (Figure 2B). This suggests that \( p53 \) might play little or no role in the regulation proliferation or apoptosis in the adult olfactory bulb cells in induced oxidative stress.

We then examined the expression of \( Bax \) in the granule and peri-gglomerular cell layers to determine the involvement of \( Bax \) versus \( p53 \) in oxidative stress driven cell proliferation or cell death (Figure 3A and 3B). We observed a significant decrease in the \( Bax \) expression at 10 mg/Kg treatment versus the control (\( P<0.01 \)) and the 20 mg/Kg treatment (\( P<0.01 \)) (Figure 3B). This also correlated with an increase in cell proliferation in total cell count (10 mg/Kg; Figure 1A and 1B). In all instances, the \( p53 \) and \( Bax \) levels did not change significantly for the control and 20 mg/Kg treatment (Figure 4). These findings suggest that \( p53 \) is relatively constant irrespective of the oxidative stress threshold and \( Bax \) acts independently in a dose-specific manner to regulate neuronal survival in the olfactory bulb. A lower dose (10 mg/Kg BW) induced proliferative changes and caused a reduction in the expression of \( Bax \) and with no corresponding change in \( p53 \) expression while 20 mg/Kg BW caused no significant change in \( Bax \), \( p53 \) and cell count when compared to the control (Figure 4).

**Discussion**

Taken together the findings of this study suggest the central role of \( Bax \) in determining oxidative stress induced proliferation or degeneration in the adult olfactory bulb. We have also shown that \( Bax \)-expression is dependent on the threshold on the induced stress while \( p53 \) remained unchanged irrespective of the stress threshold. The expression of \( p53 \) and \( Bax \) in the olfactory bulb also correlated to the outcomes of the total cell count. Cell proliferation was observed in the 10 mg/Kg treatment (Figure 1A).
Fig. 1: General histology of the olfactory bulb shown in hematoxylin and eosin staining. (A) Demonstration of the granule (G) and periglomerular (P) cell layers of the olfactory bulb in the treatment and control (Magnification X400). (B) An increase in cell number was observed in the 10 mg/Kg (**P<0.001) and not the 20 mg/Kg (NS) treatment when compared with the control. No significant change in cell count was found between the treatment groups (NS) [error bars represents SEM].

Fig. 2: Immunohistochemical localization of the P53(+) cells in the olfactory bulb. (A) Although a change in cell count was observed (Figure 1A–B), no significant change was recorded in the expression of P53 in the treatment groups versus the control. This suggests that nitric oxide/ROS induced P53 increase might not be a mechanism for oxidative stress induced cell death in the olfactory bulb. (B) No dose dependency was also observed when the 10 and 20 mg/Kg treatments were compared. Significance was set at P<0.05 for all comparisons; NS-not significant [error bars represents SEM].
Fig. 3: Bax Immunostaining (A) Photomicrographs showing the distribution of Bax (+) cells in periglomerular (P) and granule (G) cell layers of the olfactory bulb (Magnification X250). (B) A reduction in Bax expression was seen in the 10 mg/Kg treatment which was significant when compared to the control (**P<0.01) and the 20 mg/Kg (**P<0.01). This also corresponded to an increase in cell count observed in general histology (Figure 1B). A decrease in the expression of Bax indicates more cells are allowed to continue the cell cycle, thus supporting proliferation in the adult olfactory bulb (seen in the 10 mg/Kg treatment). The 20 mg/Kg treatment caused no change in Bax expression and also caused no change in cell count (Figure 1B) [error bars represents SEM].

Fig. 4: A scatter plot showing the expression of p53 and dose-dependent expression of Bax in the olfactory bulb after induced oxidative stress (n = 7). In the control and 20 mg/Kg treatment, the levels of p53 and Bax expression were unchanged. This corresponded to no significant increase in total cell count (Figure 1A and 1B). The 10 mg/Kg treatment recorded a decrease in Bax and no change in p53; corresponding to an increase in cell count [Error bars represent SD].

The interplay between p53 and Bax has long been described in the cell viability and apoptosis. Recent studies have shown that an increase in p53 or Bax often corresponds to reduce cell viability through the modulation of calcium signaling in mitochondria-linked apoptosis. Bax is central to the “suggested” p53-mitochondria apoptotic pathway by directing ROS-induced calcium-shift through its ability to increase mitochondria membrane permeability. A major comparison is the role of Bax as a calcium transport regulator in oxidative stress versus its role as a cell cycle exit protein in death of olfactory bulb neurons (apoptosis). First, an increased level of Bax in the cell cycle implies an increase in number of cells exiting the cell cycle (apoptosis) and reduction of Bax expression will promote cell viability.
and proliferation (retaining more cells in the cell cycle). This has been shown in vitro as cell proliferation increased following the use of pharmacological inhibitors of \textit{Bax}\textsuperscript{-/-}, and \textit{in vivo} in genetic deletion rodent models (\textit{Bax}\textsuperscript{-/-}).\textsuperscript{39} Second, following the release of Cytochrome C in cyanide induced oxidative stress \textit{Bax} activates \textit{Bid} signaling (t\textit{Bid}) to facilitate the release of mitochondria calcium into the cytoplasm causing autophagy.\textsuperscript{19,20} This is achieved through the kiss and run mechanism of both \textit{Bax} and t\textit{Bid} in increasing mitochondria membrane permeability (ψ\textsubscript{m}). Studies have shown that t\textit{Bid} runs faster if it has been kissed by \textit{Bax}, thus an elevated \textit{Bax} in oxidative stress indicates calcium based excitotoxicity through a mitochondria pathway.\textsuperscript{38,41} Thus, either as a cycle exit protein or calcium-shift inductor, \textit{Bax} modulates the survival of neurons; in the former through apoptosis and the later through calcium toxicity/autophagy.

The actual relationship between \textit{p53} and calcium signaling is vague. Although high levels of \textit{p53} have been reported to correspond to a increase in cerebral calcium, the central role of \textit{Bax} in Ca\textsuperscript{2+}–\textit{P53} cross talk remains elusive.\textsuperscript{42,43} In oxidative stress, it is known that ROS formation facilitates the formation of NO which can raise the nuclear level of \textit{P53} and induce apoptosis. Further, the ROS also facilitates “ROS-dependent calcium” release from the mitochondria through the Cytochrome C-\textit{Bax-t\textit{Bid}} signaling pathway.\textsuperscript{38} We can deduce form these points that ROS-induced NO production and Cytochrome C release are inducers of \textit{P53} and \textit{Bax} increase respectively. Thus, associated calcium-shift in ROS-linked elevated \textit{P53} can be said to be \textit{Bax}-dependent rather than being induced through an increased expression of \textit{P53}.\textsuperscript{34,38}

Our findings suggest that \textit{Bax} is not dependent on \textit{P53} in modulating oxidative stress and neuronal cell viability in the adult olfactory bulb (Figure 4). Furthermore, the extent of \textit{Bax} inhibition was dependent on the threshold of the oxidative stress (Figure 3A, 3B and Figure 4) while \textit{P53} showed no significant change when compared to the control. A decrease in \textit{Bax} also caused an increased cell proliferation in the olfactory bulb raising the question as to role of \textit{P53} in cell viability in the adult olfactory bulb. Other studies have reported the role of \textit{P53} in cell proliferation in the developing olfactory bulb such that genetic deletion of \textit{P53} increased number of the cells of the SVZ.\textsuperscript{7,34} However, in the adult bulb, genetic deletion \textit{P53} caused no significant increase in cell proliferation\textsuperscript{46} while genetic deletion or up regulation of \textit{Bax} altered the olfactory bulb density significantly;\textsuperscript{36} further supporting the importance of \textit{Bax} in neuronal viability in oxidative stress.

\textbf{\textit{Bax regulates neuronal survival and viability in the adult olfactory bulb}}

Adult neurogenesis has been described in the olfactory bulb. It involves the repopulation of neuronal stem cells migrating from the SVZ into the granule and periglomerular cell layers of the olfactory bulb to participate on the formation of inter neurons required for the integration of newly formed cells of the olfactory circuit.\textsuperscript{1,44} Despite the importance or neuronal survival in the olfactory bulb, the regulatory mechanism remains elusive. Hypothetically, the prominent players marked for study are the cell cycle and apoptosis protein- \textit{P53} and \textit{Bax}. Our study has shown that \textit{Bax} is more implicated for such mechanistic regulation of survival in the adult bulb while \textit{P53} relatively maintains a passive role. Our findings are further supported by previous studies which showed that genetic deletion of \textit{P53} caused no change in cell count\textsuperscript{3} while the deletion of \textit{Bax} changed the cell count signficantly \textit{in vivo}.\textsuperscript{44} \textit{Shi et al.} (2005) showed that regulatory activities of \textit{Bax} in neuronal viability of the olfactory bulb is dependent on its modulation of calcium through IP\textsubscript{3} signaling and alteration of mitochondria membrane permeability.\textsuperscript{38,45} Impairment of proliferation through upregulation of \textit{Bax} has been associated with a decrease in olfactory function and olfactory bulb volume, specifically the loss of the granule and peri-glomerular cells.\textsuperscript{45,34} The \textit{Bax}-dependent mechanism of cell loss has also been described in the olfactory bulb of rodent models of depression.\textsuperscript{46}

Although a strong relationship exists between \textit{P53} and \textit{Bax}, this study (and others) suggests that \textit{Bax} is central to the control of both proliferation and cell death in the adult olfactory bulb when compared with \textit{P53}. Also, we have demonstrated that \textit{Bax} modulation of neuronal survival in oxidative stress is dependent on the threshold of the induced-stress and together (that is \textit{Bax} expression and stress threshold) regulates the survival of neurons in the adult olfactory bulb. We deduce that it is reflective of the regional cytotoxic pathway in the olfactory bulb and how threshold of assault creates varying effects in different parts of the brain. This is an important premise for future studies on the role oxidative stress in the regulation of the activities of \textit{Bax} and how oxidative stress thresholds affects calcium signaling in the olfactory bulb relative to its cell cycle pattern.

It is in continuation to various previous studies which highlight the role of oxidative stress in neurodegenerative disorders.\textsuperscript{47–51} Orally administered KCN between 4 to 22 mg/Kg BW per day represents the sub-lethal dose for rats while 15–30 days is termed “short-term” exposure.\textsuperscript{52} We anticipate variations in the findings for varied doses and duration. We have selected this treatment algorithm as it is representative of human exposure from cyanophoric plant diets.\textsuperscript{53,54}

\textbf{Conclusion}

Although \textit{P53} play an important role in development of the olfactory bulb, our findings suggest that it has little contribution in neuronal cell viability and proliferation in the adult olfactory bulb. No significant change in \textit{P53} was observed irrespective of treatment dose and cell count while \textit{Bax} expression was reduced at 10 mg/Kg treatment and was associated with an increased cell count. We conclude that regulation of survival of neurons in the adult olfactory bulb, following induced-oxidative stress was more dependent of the expression of \textit{Bax} and the treatment concentration rather than \textit{P53} expression.

\textbf{Authorship Contributions}

Olalekan M Ogundele: Main author and investigator that designed the experiments and wrote the manuscript, Olurotimi J Sanya: Participated in the revision of the manuscript and analysis of the result.

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