General Discussion

Cigarette smoke-induced pathophysiology involves several processes that collectively lead to tissue damage. One of the most prominent effects of cigarette smoking is oxidative damage. An estimated $10^{17}$ stable long lived radicals per gram of tar phase and $10^{15}$ radicals per puff of gas phase are present in cigarette smoke (Pryor 1993). But results from our laboratory indicate that gas phase radicals are extremely unstable and unable to produce oxidative protein damage. Rather, tar phase radicals are responsible for cigarette smoke-mediated oxidative damage (Panda 2001).

The tar phase long lived radicals contain semiquinones (Pryor 1998). The oxidative damage done by the tar phase semiquinones was proposed to be carried out by redox cycling involving formation of reactive oxygen species (ROS) (Pryor 1998). However, we had reported that the semiquinone present in the tar phase is largely p-benzoquinone (p-BSQ), which is a strong reducing agent. Under physiological condition, p-BSQ is converted to p-benzoquinone (p-BQ) by disproportionation (Banerjee 2008). In the lungs of smokers, p-BSQ is oxidized to p-BQ by transition metal containing proteins (Banerjee 2008). P-BQ is a strong arylation agent. It forms covalent Michael adduct with the ε-amino groups of side chain lysine residues of proteins resulting in alterations of structure and function (Banerjee 2008). P-BQ is also a strong oxidizing agent and causes oxidative damage of proteins through redox cycling and ROS formation (Ghosh 2012, Valvanidis 2009).

In the present study, we show that exposure to CS leads to the formation of p-BQ-protein adducts in smokers blood serum albumin. The p-BQ formed in the lung of the smokers reaches the blood and forms p-BQ adduct with serum albumin (HSA) (Ghosh 2012). Cigarette smoke mediated p-BQ-adduct formation has been further validated by studies with marginal ascorbate-deficient cigarette smoke-exposed guinea pigs, where p-BQ derived from CS forms adduct with lung proteins and also serum albumin (Ghosh 2012).

Using human serum albumin (HSA) as a model protein, we have further demonstrated that after formation of adduct with p-BQ the structure and function of HSA are altered (Ghosh 2012). The secondary and tertiary structure is severely affected and protein became more...
compact (Ghosh 2012). The effect of p-BQ on functional aspects of the protein has been revealed by ligand binding assays that confirmed that fatty acid and drug binding capacity, with particular respect to quercitin and paracetamol, are significantly affected (Ghosh 2012). One such effect would be impairment of fatty acid binding and transport resulting in accumulation of lipid in the blood. This is probably one of the mechanisms of smoke-related atherosclerosis.

Our previous studies had indicated that cigarette smoke exposure leads to oxidative stress, inflammation and apoptosis. Using marginal ascorbate-deficient guinea pigs, we have observed that cigarette smoking leads to progressive lung damage indicating emphysematous lesion development (Banerjee 2007; Banerjee 2008). Previous studies elsewhere reported development of emphysema in animal models like rats (Lee 2005) and mice (Martorana 2008). These animals are capable of synthesizing vitamin C (Chatterjee 1973). Since vitamin C prevents oxidative damage and emphysema, it took months and even up to a year for the development of emphysematous lesion. Even in guinea pigs, it took a fairly long time (Wright 1990). Probable reason for such long duration is that the authors did not restrict dietary vitamin C in the guinea pigs. We have developed a marginal vitamin C-deficient guinea pig for our studies. Marginal ascorbate-deficient animals have been given vitamin C at a dose that prevents the onset of scurvy but maintains normal physiological activities (King 1940). Marginal ascorbate-deficient animals also mimic human smokers in the respect that plasma vitamin C levels are markedly low (Das 2012). The procedure of smoke exposure we adopted typically simulates human smoking (Panda 2000).

Our studies indicate that the initial effect of cigarette smoking is oxidative damage of the lung as evidenced by the formation of protein carbonyls (Panda 1999; Panda 2000; Panda 2001; Banerjee 2007; Banerjee 2008). The current thesis further confirms the earlier observations that cigarette smoke exposure increases protein carbonyls in a time dependent manner. The oxidative damage is further indicated by DNA oxidation, as evidenced by the formation of 8-hydroxy-2'-deoxyguanosine (80HdG). The amount of p-BSQ entering the lung by cigarette smoke exposure was calculated and the amount of p-BQ generated from p-BSQ entering the lung under the present experimental condition was also calculated. The p-BQ in equivalent amounts derived from CS was injected to marginal ascorbate-deficient guinea pigs. It has been observed that the formation of p-BQ adduct with lung proteins increased in a time dependent manner as evidenced
by the appearance of protein carbonyls and 8OHdG. An important component of emphysema development is inflammation and CS exposure was previously found to induce infiltration of neutrophils in alveolar cells and septal regions (Banerjee 2007). The BALF and lung also showed an increased MMP level confirming elevated protease activity (Banerjee 2008). The present study also indicates increased levels of inflammatory MMPs and degradation of elastin protein indicating loss of lung elastic recoil property. Treatment with p-BQ also resulted in the enhancement of MMP levels that was accompanied by the decrease in elastin level confirming the role of p-BQ in inducing inflammatory response within the lung.

Another important pathological process underlying the development of emphysema is apoptotic cell death (Tuder 2003). Here we show that oxidative protein damage is followed by apoptosis, which is accompanied by lung damage. Apoptosis is a tightly regulated mechanism of cell death, a process by which the cells eliminate unwanted damaged cells. The markers of apoptosis include DNA fragmentation, activation of caspases, particularly caspase 3 and caspase 8, and over expression of the proapoptotic Bax. Caspases are aspartate-directed cysteine proteases with a pivotal role in apoptosis. Caspases are initially synthesized as polypeptide chains that undergo cleavage. Cleaved caspases become activated and execute apoptosis. The apoptotic signals in mammals may be transduced by “intrinsic” and/or “extrinsic” pathways. The intrinsic pathway is primarily activated in by oxidative stress that incite increased permeability of mitochondrial membranes, release of cytochrome c from the mitochondria to the cytosol, and activation of caspase 3, a key effector protein in the execution of downstream event in apoptosis. Extrinsic apoptotic pathway involves transduction of a signal from membrane receptors belonging to the tumor receptor factor such as Fas or TNF receptor 1. Stimulation of death receptors results in activation of caspase 8, which in turn activates caspase 3. We have shown in this thesis that exposure of CS to marginal vitamin C-deficient guinea pigs induce both “intrinsic” and “extrinsic” pathways of apoptosis in a time-dependent manner. Similar observations were made when CS exposure was replaced by treatment with p-BQ. It is known that the ratio of Bax and Bcl-2 determines whether a cell will undergo apoptotic death or not. In our guinea pig model of CS-induced lung damage, here we demonstrate a marked increase in DNA fragmentation (increase in TUNEL positive cells), activation of caspase 3 and caspase 8. There was a rise in the level of the proapoptotic Bax protein, but no rise in the level of the antiapoptotic Bcl-2, resulting in an increase of the Bax/Bcl-2 ratio.
Previously we had indicated that CS causes oxidative damage to guinea pig lung proteins both in vitro and in vivo that was prevented by vitamin C (Panda 1999; 2000). Here we show that administration of a moderately large dose of vitamin C (15 mg/guinea pig /day) not only prevents the oxidative protein damage, but also apoptosis and lung lesions both in CS-exposed guinea pigs as well as p-BQ-treated animals. However, once the lung is damaged, neither administration of vitamin C nor discontinuation of the smoke exposure reverses the lung injury. Vitamin C is a strong reducer of p-BQ. It is considered that prevention of CS-induced damage is mainly due to reduction and thereby inactivation of p-BQ. Vitamin C prevents CS/p-BQ-induced protein oxidation and subsequent apoptosis and emphysema. In CS-exposed guinea pigs, a moderately large dose of vitamin C (15mg/day) is needed in order to maintain adequate tissue level of vitamin C. This is because CS consumes vitamin C (Panda 1999) and a maintenance dose of 1mg/day or 5mg/day is inadequate to sustain sufficient tissue vitamin C content after smoke exposure.

Until now there is no novel or even currently effective treatment aimed at emphysema, an irreversible fatal disease (Tuder 2006). An alternative way would be prevention of emphysema. Since the strongest risk factor of emphysema is cigarette smoking, the simplest method of prevention of emphysema would be cessation of smoking. However, this has proven difficult to achieve. So, the alternative strategy would be to identify and remove or inactivate the primary cause of CS-related emphysema. This would minimize the huge medical expenses involved, premature death and also heavy loss in productivity due to missed work. In this thesis, we have shown that p-BQ is a major factor for producing CS-induced emphysema in guinea pigs. The mechanisms of CS-induced emphysema are mimicked by p-BQ. The sequence of events in both the cases is oxidative damage, inflammation, apoptosis, ultimately leading to emphysema. We have also shown that a moderately large dose of vitamin C prevents all the pathophysiological events including emphysema. We have used marginal vitamin C-deficient guinea pig, which is obviously a better animal model to study the pathophysiology and molecular mechanisms of CS-induced emphysema. The guinea pig, like human, is incapable of synthesizing vitamin C and is dependent on the dietary source of the vitamin (Chatterjee 1973). Moreover, the guinea pig has anatomical and CS-induced pathophysiological similarities to human (Wright 2002). We consider that the results obtained with guinea pigs would be applicable to humans. In that case,
intake of moderately large doses of vitamin C might protect the smokers from emphysematous lung damage, as indicated in the following scheme (Figure 1).

\[
\begin{align*}
\text{CS} & \rightarrow \text{p-BSQ} \rightarrow \text{p-BQ} \\
& \downarrow \text{Vitamin C} \\
\text{Lung} & \rightarrow \text{Oxidative damage} \rightarrow \text{Inflammation} \rightarrow \text{Apoptosis} \rightarrow \text{Emphysema}
\end{align*}
\]

Figure 1 Molecular mechanism of cigarette smoke-induced emphysema and prevention by vitamin C.