Chapter VII

Glycyrrhizic acid modulates NF-κB suppression, caspases activation and cytotoxicity induced by benzo(a)pyrene exposure in rat lungs: probable role of soluble epoxide hydrolase and thioredoxin reductase
Introduction

Glycyrrhizinic acid, a triterpenoid saponin glycoside, is an active principle of *Glycyrrhiza glabra* (Licorice). It is widely applied as a sweetener in food and tobacco products (Ploeger et al., 2001). In traditional systems of medicine licorice is used to treat various lung ailments. In various studies glycyrrhizic acid is reported to alter inflammatory processes by modulation of NF-κB activities (Schröfelbauer, 2009).

Benzo(a)pyrene [B(a)P], a prototype of polynuclear aromatic hydrocarbons (PAHs) family, is formed during the process of incomplete combustion of organic matter such as fossil fuel, garbage and plant parts. It is also present in tobacco smoke (Piccardo et al., 2010). B(a)P is an environmental contaminant and known to cause various toxicities including airway inflammation and injuries (Podechard et al., 2008). In short term exposures B(a)P is reported to alter cellular antioxidant levels (Lina et al., 2007), an initial role in oxidative status of the cell besides its known carcinogenic activities. Such kind of exposures can play an instrumental role in alterations of lung architecture and physiology that may lead to development of various lung disorders including chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, emphysema and lung cancer. B(a)P has been proved as a model
toxicant to study adverse effects on pulmonary system in Wolterbeek et al., 1995). In the present study B(a)P was used to study the alterations in NF-κB, a transcription factor that controls expression of numerous genes involved in immunological processes and cell survival, in relation with activities of soluble epoxide hydrolase (sEH) and thioredoxin reductase (TrxR) in lungs of Wistar rats. sEH is known to play a major role in cardiovascular diseases and possess proinflammatory properties. sEH is responsible for metabolizing epoxycicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids (DHETs). Inhibition of sEH is reported to reduce inflammation due to elevated levels of EETs (Schmelzer, 2005). These activities could be due to NF-κB inhibiting properties of EETs (Node, 1999). TrxR is a selenocysteine containing flavoprotein and has an ability to maintain thioredoxin (Trx) in reduced state, which regulates the activity of NF-κB. Thus TrxR plays a role in redox regulation of NF-κB (Hayashi et al., 1993; Sakurai et al., 2004). Interference with activities of sEH and TrxR by B(a)P can affect NF-κB activities and dependent immunological alterations and cell survival. On the basis of these facts the present study was designed to assess protective activities of glycyrrhizic acid against B(a)P induced lung debilities in Wistar rats. Doses of the glycyrrhizic acid were selected on the basis of previously published reports (van Gelderen et al., 2000; Tanahashi et al., 2002); keeping in mind that higher dose may cause toxic responses (Cantelli-Forti et al., 1997).

Treatment regimen

To study the effect of pre-treatment of animals with glycyrrhizic acid on B(a)P induced lung epithelial damages, 24 male Wistar rats were randomly allocated to four groups of six rats in each. The animals of Group I & II served as control and toxicant [B(a)P] respectively, received vehicle (0.15 M NaCl) orally (5 ml/kg b.wt., once daily, for 14 days). Group III received pre-treatment with glycyrrhizic acid by gavages once daily for 14 days at a dose of
50 mg/kg b.wt. (GAI). Group IV received glycyrrhizic acid once daily for 14 consecutive days at a dose level of 100 mg/kg b.wt. (GAI). On day 12 and 14, one hour after the treatment with glycyrrhizic acid or vehicle the animals of Group II, III and IV were administered with an intratracheal dose of B(a)P (5 mg/kg b.wt. in 1% gelatin in 0.15 M NaCl), Group I (control group) was administered with 1% gelatin intratracheally. All the animals were sacrificed 24 h after last treatment.

Results

**Soluble Epoxide Hydrolase activity and NF-κB translocation**

Soluble epoxide hydrolase (sEH) activity was found to be significantly reduced ($p<0.05$) after intratracheal B(a)P exposure in Group II when compared with Group I (Fig 1a). Pre-treatment with GAI and GAI (glycyrrhizic acid 50 and 100 mg/kg b.wt. respectively), in Group III and IV respectively, show significant protection of sEH activity when compared with Group II (Fig 1a). NF-κB content translocated to nucleus was decreased by B(a)P exposure ($p<0.05$) when compared with Group I (Fig. 1b). Pre-treatment with GAI and GAI, in Group III and IV respectively, show significant increase ($p<0.01$) in translocated NF-κB when compared with Group II (Fig. 1b).

**$H_2O_2$ generation, Catalase and Thioredoxin Reductase (TrxR) activities**

$B$(a)P administration in Group II animals showed significant enhancement in levels of $H_2O_2$ in lung tissue when compared with Group I (control group) (Table 1). Pre-treatment with GAI and GAI, in Group III and IV, showed a significant decrease ($p<0.05$ and $p<0.01$ respectively) in $H_2O_2$ generation. Moreover, intratracheal instillation of B(a)P in Group II caused a significant decrease in the activities of TrxR and catalase ($p < 0.05$) when compared
with Group I (control) animals (Table 1). Administration of GAI (glycyrrhizic acid 50 mg/kg b.wt.) + B(a)P in Group III animals showed a significant increase in catalase activity ($p<0.05$) but changes in TrxR activity were not found to be significant when compared with Group II (B(a)P-treated animals). However GAII (glycyrrhizic acid 100 mg/kg b.wt.) (Group IV) showed a significant increase ($p<0.05$) in activities of catalase and TrxR when compared with Group II.

**TNFα and IFNγ levels**

B(a)P administration, in Group II, significantly suppresses ($p<0.05$) levels of TNFα and IFNγ in lung tissue when compared with Group I (Fig. 2a and 2b respectively). Pre-treatment with GA1 and GAII, in Group III and IV, did not prove any significant alterations in TNFα and IFNγ levels when compared with Group II (Fig. 2a and 2b).

**Caspases activation and necrotic cell death**

Intratracheal administration of B(a)P caused a significant increase ($p<0.01$) in caspases activities (Caspase -2, -3, -6, -8, -9) when compared with Group I (Fig. 3). GA I in Group III did not show any significant alterations when compared with Group II. However GA II in Group IV exhibited significant reduction ($p<0.01$) in activities of all the caspases (Fig. 4). B(a)P exposure in Group II animals showed significant enhancement in levels of cytotoxicity markers LDH ($p<0.01$) and ALP ($p<0.05$) (Fig. 4a and 4b respectively) in BALF when compared with Group I. Group III and IV on glycyrrhizic acid pre-treatment showed a significant decrease in LDH and ALP indicating protective effect of glycyrrhizic acid against
B(a)P induced cytotoxicity/necrotic events in lungs when compared with Group II (Fig. 4a and 4b respectively).

Discussion

Glycyrrhizic acid is reported to alter the activities of various gene products and expression of the genes itself in a variety of pathological conditions (Kao et al., 2010; Tu et al., 2010). In this way it shows its potential to counter the effects of toxicants, in animals and humans, affecting the physiological processes at molecular and biochemical levels. In the present study repeated lung exposure to B(a)P shows damaging effects in lung epithelium by suppression of NF-κB and induction of oxidative stress in lungs of Wistar rats. Inhibition of NF-κB and downstream pathways and generation of ROS can lead to apoptotic cell death (Li et al., 2002) and tissue injury. Moreover B(a)P is reported to induce apoptotic events in rat lungs when exposed intratrachcally or intra-pulmonary (Gosset et al., 2003; Silva et al., 2010). Epithelial damages were very much obvious in terms of elevated levels of ALP and LDH activities in BALF, and caspases activities in lung tissue. TNFα, a proinflammatory cytokine, plays a critical role in the activation of NF-κB (Karin et al., 2000) was found to be suppressed by B(a)P exposure. IFNγ, which is reported to work synergistically with TNFα to enhance the binding of NF-κB with DNA (Yasumoto et al., 1992) was found to be inhibited as well (Group II). Initially, in the present investigation, it appears that NF-κB inhibition is probably due to suppression of TNFα by B(a)P exposure. This kind of alterations in cytokine levels and suppression of TNFα by B(a)P is also reported in studies elsewhere (Kong et al., 1994; Schellenberger et al., 2009). In various immunological conditions NF-κB activation has been found to be dependent on TNFα release, and suppression of this cytokine can directly affect activation and translocation of NF-κB into nucleus. However glycyrrhizic acid, in
present investigation, appears to reverse the suppression of NF-κB in a manner that is independent of TNFα release. It is not altering the levels of TNFα. Hence it may be justified by the altered activities of two intracellular enzymes, soluble epoxide hydrolase (sEH) and thioredoxin reductase (TrxR), in all the treatment groups. Both the enzymes are reported to alter NF-κB activities (Norwood et al., 2010; Sakurai et al., 2004). Suppression of sEH activity can result in accumulation of epoxyeicosatrienoic acids (EETs) which is known to suppress NF-κB (Fang et al., 2001) and downstream expression of various genes involved in cell survival and immunological processes. Suppression of sEH activity and TNFα by B(a)P (in Group II) may provide a mechanism involved in suppression of NF-κB and modulation of cell survival activities and consequent epithelial injuries. Moreover, B(a)P was found to inhibit TrxR activities as well, and glycyrrhizic acid exhibited protection of this enzyme (Group III and IV). These changes were found to be correlated with induced level of hydrogen peroxide by B(a)P that was eventually suppressed by glycyrrhizic acid. Hydrogen peroxide may play a role in inhibition of TrxR activities (Arner et al., 2000). TrxR activity is essential for NF-κB which is well known for its redox sensitivities (Flohé et al., 1997). TrxR maintains thioredoxin, a thiol containing protein, in its reduced form that is necessary for NF-κB binding with DNA (Flohé et al., 1997). Inhibition of TrxR activities is also related with cytotoxicity induction (Kurosawa et al., 2009). Modulation of TrxR activities can provide an insight about the altered status of NF-κB. B(a)P (in Group II) was found to suppress NF-κB and cell survival probably by suppressing TNFα release, and inhibition of sEH and TrxR activities. However, results indicate that reversal of NF-κB suppression by glycyrrhizic acid is independent of TNFα activities. Figure 5 shows the proposed mechanism involved. These findings may provide an insight into the mechanisms involved in alteration of NF-κB activities and tissue injuries by B(a)P and probably other members of PAHs family.
In conclusion, results indicate an involvement of inhibition of sEH and TrxR, TNFα suppression and induction of oxidative stress and consequent lung injuries by short term exposure of B(a)P. Glycyrrhizic acid shows its potential against these toxic manifestations by a mechanism probably involving alterations in sEH and TrxR, NF-κB induction and suppression of oxidative stress in lungs of Wistar rats. Glycyrrhizic acid may have modulatory effects against other lung pathological conditions arising due to inflammatory processes.
Table 1: Effect of B(a)P and glycyrrhizic acid on intracellular antioxidant enzymes thioredoxin reductase and catalase, and hydrogen peroxide level in lung tissue.

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<tr>
<th>Treatment Groups</th>
<th>Lung Tissue</th>
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<tr>
<td></td>
<td>Thioredoxin reductase (nmol DTNB reduced/min/mg protein)</td>
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<tr>
<td>I. Control</td>
<td>234.56 ± 16.6</td>
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<tr>
<td>II. B(a)P, 5 mg/kg b.wt. (i.t.)</td>
<td>156.66 ± 12.7&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>III. GA (50 mg/kg b.wt.)</td>
<td>163.08 ± 10.7&lt;sup&gt;NS&lt;/sup&gt;</td>
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<tr>
<td>IV. GAII (100 mg/kg b.wt.)</td>
<td>218.00 ± 29.8&lt;sup&gt;*&lt;/sup&gt;</td>
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Values are means ± S.D. (n = 6). Significant differences are indicated by <sup>†</sup>p < 0.05 when compared with control animals (Group I), <sup>*</sup>p < 0.05 and <sup>**</sup>p < 0.01 when compared with B(a)P-treated animals (Group II), NS is not significant. GA = Glycyrrhizic acid, B(a)P = Benzo(a)pyrene.
Figure 1: Effect of B(a)P and glycyrrhizic acid on (a) Soluble Epoxide Hydrolase activity in lung tissue cytosolic fraction and (b) NF-κB level in nuclear fractions.

Values are means ± S.D. (n = 6). Significant differences are indicated by #p<0.05 when compared with control animals (Group I), *p<0.05 and **p<0.01 when compared with B(a)P-treated animals (Group II). Group III = Glycyrrhizic acid (50 mg/kg b.wt.), Group IV = Glycyrrhizic acid (100 mg/kg b.wt.).
Figure 2: Effect of B(a)P and glycyrrhizic acid on (a) TNFα and (b) IFNγ levels in lung.

Values are means ± S.D. (n = 6). Significant differences are indicated by #p<0.05 when compared with control animals (Group I); NS = not significant when compared with B(a)P-treated animals (Group II). Group III = Glycyrrhizic acid (50 mg/kg b.wt.), Group IV = Glycyrrhizic acid (100 mg/kg b.wt.).
Figure 3: Effect of B(a)P and glycyrrhizic acid on caspases (-2, -3, -6, -8 and -9) activities in lung tissue.

Caspases activity

Values are means ± S.D. (n = 6). Significant differences are indicated by **p<0.01 when compared with control animals (Group I); *p<0.01 when compared with B(a)P-treated animals (Group II); NS = not significant when compared with Group II. Group III = Glycyrrhizic acid (50 mg/kg b.wt.), Group IV = Glycyrrhizic acid (100 mg/kg b.wt.).
Figure 4: Effect of B(a)P and glycyrrhizic acid on lung epithelial damage markers (a) LDH and (b) ALP in bronchoalveolar lavage fluid (BALF).

![Graphs showing LDH and ALP levels across different treatment groups.](image)

Values are means ± S.D. \((n = 6)\). Significant differences are indicated by \(\# p<0.05\) and \(\#\# p<0.01\) when compared with control animals (Group I), \(* p<0.05\) and \(*\# p<0.01\) when compared with B(a)P-treated animals (Group II). Group III = Glycyrrhizic acid (50 mg/kg b.wt.), Group IV = Glycyrrhizic acid (100 mg/kg b.wt.).
Figure 5: Proposed Mechanism of Glycyrrhizic acid against acute Benzo(a)pyrene induced NF-κB suppression and cell death in rat lungs

Figure: B(a)P = Benzo(a)pyrene; ROS = Reactive oxygen species; TNFα = Tumor necrosis factor alpha; NF-κB = Nuclear factor kappa B; sEH = soluble epoxide hydrolase; TrxR = Thioredoxin reductase; Trx = Thioredoxin; EET = Epoxycosatrienoic acid; DHET = Dihydroxy epoxycosatrienoic acid; IκB = Inhibitor of kappa B; IKK = Inhibitor of kappa B kinase; H₂O₂ = Hydrogen peroxide; HO• = Hydroxyl radical.