ELSEVIER

Biochimica et Biophysica Acta 1536 (2001) 21–30

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Xanthine oxidase-derived reactive oxygen metabolites contribute to liver necrosis: protection by 4-hydroxypyrazolo[3,4-d]pyrimidine

Shakir Ali [a,*], G. Diwakar [a], Sonica Pawa [a], M.R. Siddiqui [a], M.Z. Abdin [b], F.J. Ahmad [c], S.K. Jain [b]

[a] *Department of Biochemistry, Faculty of Science, Hamdard University, New Delhi 1100 62, India*
[b] *Center for Biotechnology, Hamdard University, New Delhi 1100 62, India*
[c] *Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi 1100 62, India*

Received 2 October 2000; received in revised form 25 January 2001; accepted 5 February 2001

Abstract

Xanthine oxidase (XO) generates reactive oxygen metabolites (ROM) as a by-product while catalyzing their reaction. The present study implicates these ROM in the pathogenesis of liver necrosis produced in rats by the intraperitoneal administration of thioacetamide (TAA; 400 mg/kg b.wt.). After 16 h of TAA administration, the activity of rat liver XO increased significantly compared to that of the control group. At the same time, the level of serum marker enzymes of liver necrosis (aminotransferases and alkaline phosphatase) and tissue malondialdehyde content also increased in TAA treated rats. Tissue malondialdehyde concentration is an indicator of lipid peroxidation and acts as a useful marker of oxidative damage. Pretreatment of rats with XO inhibitor (4-hydroxypyrazolo[3,4-d]pyrimidine; allopurinol (AP)) followed by TAA could lower the hepatotoxin-mediated rise in malondialdehyde level as well as the level of marker enzymes associated with liver necrosis. The survival rate also increased in rats given AP followed by the lethal dose of TAA. In either case, the effect of AP was dose-dependent. Results presented in the paper indicate that increased production of XO-derived ROM contributes to liver necrosis, which can be protected by AP.

Keywords: Thioacetamide; Necrosis; Oxidative damage; Anti-hepatotoxic; 4-Hydroxypyrazolo[3,4-d]pyrimidine; Xanthine oxidase

1. Introduction

Necrosis of liver is a complex process involving swelling of the cells and loss of plasma membrane integrity leading to cell lysis, and consequently to hepatic failure. Virus, drugs and chemicals are among the factors, which may lead to necrosis and subsequent liver failure [1]. Thioacetamide (TAA), a thiono-sulfur-containing compound endowed with liver damaging activity, is often used to induce experimental liver necrosis in animal species [2–4]. Shortly after administration, TAA undergoes an extensive metabolism to acetamide and thioacetamide *S*-oxide by the mixed function oxidase system [5]. The monooxygenase system further metabolizes thioacetamide *S*-oxide to thioacetamide *S*-dioxide, which is capable of binding to tissue macromolecules and is thought to produce damage [6,7]. Also, partially reduced cytotoxic oxygen moieties (reactive oxygen metabolites, ROM) have been implicated in the necrotic process induced by TAA [2,3]. The occurrence of extensive radical reactions in the liver during the necrotic process has been reported in the Wistar

* Corresponding author. E-mail: ali_shakir@hotmail.com

0925-4439/01/$ – see front matter
PII: S0925-4439(01)00030-8

strain of rat [3]. Based on the decrease of the liver concentrations of vitamins C and E and the formation of lipid hydroperoxides after TAA administration, an increase in oxidative stress by TAA has been suggested by Sun et al. [3]. The cellular source leading to excess generation of ROM after TAA exposure has, however, not been established.

ROM have been held responsible for the pathogenesis of many forms of tissue injuries. When the cells are exposed to excess ROM, oxidative damage occurs which affects many cellular functions that trigger cell death. In the present investigation, evidence is provided for the involvement of ROM in the necrosis produced by TAA. Xanthine oxidase (XO), the enzyme which generates ROM as a by-product while catalyzing their reaction, is reported here to increase in TAA treated rats and is suggested to initiate the necrotic process. Evidence is provided to establish the role of XO-derived ROM in the necrotic process, taking the hepatic malondialdehyde content as an index to evaluate oxidative damage. A variety of antioxidants scavenge free radicals and prevent oxidative damage in the cell. The primary defense against oxidative damage in the tissue rests with antioxidants such as the tripeptide glutathione (GSH), which is consumed in order to counteract the effects of oxidative stress [3]. The concentration of reduced GSH in rat liver was, therefore, determined as another type of index of oxidative stress produced by TAA. In vivo XO inhibitor, 4-hydroxypyrazolo[3,4-d]pyrimidine (allopurinol, AP), is shown to protect the tissue against TAA-induced liver necrosis in a dose-dependent manner.

2. Materials and methods

2.1. Animals and other material

Adult female albino Wistar rats (weighing 150–200 g) were obtained from and kept in the Central Animal House Facility of Jamia Hamdard in propylene cages in an environmentally controlled room with a 12 h light–dark cycle at constant room temperature (24 ± 2°C). Animals had free access to a standard pellet diet and tap water ad libitum. Guidelines issued by the Jamia Hamdard Animal Ethics Committee for the care and use of laboratory animals were followed. For experimental purpose, animals were randomly assigned to different groups, with six animals in each cage. Chemicals used in this study were of the highest purity grade available from standard commercial sources in India. Diagnostic kits were purchased from Span Diagnostics (India). TAA was procured from Sigma (St. Louis, MO, USA).

2.2. Induction of liver necrosis and its evaluation by analytical methods

Liver necrosis was induced in rats by a single intraperitoneal injection of TAA (400 mg/kg b.wt.), dissolved in saline (0.9% NaCl), as previously described [4]. Control rats received a similar volume of normal saline by the same route. Liver injury was evaluated by analyzing serum/tissue lysate obtained from the animals sacrificed 16 h after the TAA administration. Rats were anesthetized with ether and the blood was drawn by cardiac puncture. Blood was allowed to clot and the serum was separated by centrifugation. Serum was used for the analyses of aminotransferases and alkaline phosphatase levels using diagnostic kits, based on the methods of Reitman [8] and Bessey [9], respectively. Other biochemical estimations were performed on a 10% tissue homogenate (lysate) prepared from the liver. The liver of the animal was immediately removed, washed in ice-cold saline solution, blotted and a small portion was cut and weighed for homogenization. All the subsequent operations were carried out at 0–4°C.

Tissue lysate was prepared in nine volumes of 0.1 M phosphate buffer (pH 7.4) containing 1.15% KCl, using a polytron homogenizer. A portion of the lysate was kept for the determination of hepatic malondialdehyde content [10] and the content of reduced GSH [11]. The rest of the lysate was subjected to differential centrifugation in a cooling centrifuge initially at $800 \times g$ for 10 min to remove the nuclei and other cell debris, and then the resultant supernatant was centrifuged at $9500 \times g$ for 20 min to get the post-mitochondrial supernatant (PMS). The PMS was used to measure the specific activity of XO [12]. The protein content of each sample was determined according to Lowry's method [13] using bovine serum albumin as reference protein. All the biochemical estimations were completed on the day the animals were sacrificed.

2.3. Experimental design for evaluating the anti-hepatotoxic activity of AP

Anti-hepatotoxic activity of XO inhibitor AP was demonstrated at different dose levels of the drug. Various doses of AP, viz. 7.5, 15 or 30 mg/kg b.wt., prepared in distilled water, were administered to rats orally for three consecutive days (at 24 h intervals) followed by the administration of TAA (400 mg/kg b.wt., i.p.). TAA was given an hour after the last dose of AP was administered. The groups of rats receiving various doses of AP followed by TAA are referred to as $AP_{7.5}$, AP_{15}, and AP_{30}. The groups treated with TAA alone and with the highest dose of AP (30 mg/kg b.wt.) alone served as the positive control groups. All the biochemical parameters mentioned in Section 2.2 of this text were estimated in the experimental and the control group of rats sacrificed 16 h after TAA administration.

2.4. Survival studies

These experiments were performed to examine the protective effect of AP on survival in rats receiving a lethal dose of TAA (i.e. 150 mg/kg b.wt., p.o., daily, for 12 consecutive days). Animals for these studies were divided into five groups, each containing 10 rats. Three groups were given orally different doses of AP (7.5, 15 or 30 mg/kg b.wt., for three consecutive days) followed after 1 h by the administration of the lethal dose of TAA. The fourth and the fifth groups were given, respectively, the lethal dose of TAA alone and the highest dose of AP alone. Mortality in each group was recorded daily for a week after the last dose of TAA was administered. Nine out of 10 rats died in the fourth group (the group given the lethal dose of TAA), while no mortality was observed in the fifth group (i.e. the group receiving 30 mg AP/kg b.wt.). Results are expressed as the number of rats that survived in each group.

The protective effect of XO inhibition on survival was further investigated using tungsten. Tungsten has a molybdenum-antagonistic effect and, when gavaged to rats as sodium tungstate (ST), it inhibits the activity of XO, which is a molybdoprotein. Animals were gavaged with ST (50 mg/kg b.wt., daily, for 7 weeks) followed by the above mentioned lethal dose of TAA. Survival was calculated 2 weeks after the last dose of TAA was administered. The group of rats receiving the lethal dose of TAA alone served as the control group. No mortality was observed in the group of rats given the same dose of ST for a similar duration of time.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. and analyzed by one-way analysis of variance combined with Newman–Keuls test. The level of statistical significance was chosen as $P < 0.05$.

3. Results

3.1. Effect of TAA on biochemical parameters associated with liver necrosis

Serum levels of alanine aminotransferase (sALT), aspartate aminotransferase (sASP) and alkaline phosphatase (sALP) were measured and found to be elevated significantly (Table 1) in rats sacrificed 16 h after receiving a necrogenic dose of TAA (400 mg/kg b.wt., i.p.). These results indicate liver injury after TAA exposure, as described previously [2,4].

Table 1
Serum levels of enzymes associated with TAA-induced liver necrosis in rats

Treatment	Biochemical markers of liver necrosis		
	sALT (IU/ml)	sASP (IU/ml)	sALP (Eq. U/ml)
Control	35.9 ± 0.83	127.7 ± 2.07	7.1 ± 0.03
Thioacetamide	113.6 ± 0.86*	182.9 ± 2.83*	9.4 ± 0.05*

Enzyme activity was determined 16 h after a single intraperitoneal injection of TAA (400 mg/kg b.wt.) as per Section 2. Control rats received saline in place of TAA. Values are means ± S.E.M. of six rats and asterisks indicate significant difference from the corresponding control group, *$P < 0.05$. sALT: serum alanine aminotransferase; sASP: serum aspartate aminotransferase; sALP: serum alkaline phosphatase.

Table 2
Hepatic XO activity, malondialdehyde content and reduced GSH level in rats receiving a single injection of TAA

Biochemical parameters	Control	Thioacetamide
Xanthine oxidase (μmol uric acid/mg protein)	320 ± 4.63	520 ± 8.57*
Lipid peroxidation (nmol malondialdehyde/g liver)	15.4 ± 1.3	22.3 ± 0.07*
Reduced GSH content (μmol/g liver)	311 ± 13.6	149 ± 15.8*

Biochemical analyses were performed on the tissue samples obtained from rats sacrificed 16 h after the administration of a single necrogenic dose of TAA according to Section 2. Control rats received normal saline instead of TAA. Values are means ± S.E.M. of six rats and asterisks indicate significant difference from the corresponding control group, $*P < 0.05$.

Hepatic malondialdehyde content also increased significantly after TAA administration to rats (Table 2) suggesting that the damage is oxidative. Oxidative damage is the result of oxidative stress produced due to the increased generation of ROM, which subsequently interfere with cellular macromolecules (such as the membrane lipids) leading to tissue injury. Formation of the aldehydic products like malondialdehyde is generally considered as a reliable indicator of oxidative stress. An increase in the malondialdehyde content of the tissue indicates the involvement of ROM in the necrotic process. Excess generation of ROM in TAA treated rats can be attributed to the increased activity of XO (Table 2), which generates ROM while catalyzing its reaction.

An increase in tissue reduced GSH level is also observed in TAA treated rats (Table 2). As already mentioned, enhanced radical reactions are expected to consume the endogenous tissue antioxidants such as reduced GSH, thereby depleting its concentration within the tissue. Tissue GSH level can, therefore, act as another type of index of oxidative damage [3]. Depletion in the hepatic GSH concentration suggests the involvement of oxidative stress in the pathogenesis of liver necrosis produced by TAA. Correlating to the changes in the malondialdehyde level of the tissue (which increased after TAA treatment), a decrease in the hepatic GSH concentration was observed in TAA treated rats (Table 2). This seems to be the result of an effort by the tissue to counteract

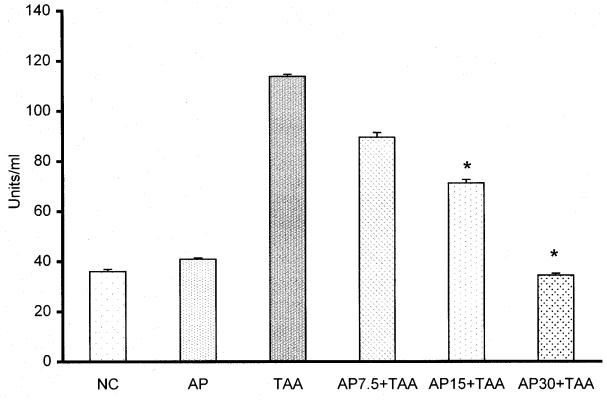

Fig. 1. sALT level in the rats treated with AP followed by a single necrogenic dose of TAA. The value of AP alone shown in the figure is the value obtained from the group of rats receiving a dose of 30 mg/kg b.wt. for three consecutive days. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of TAA (400 mg/kg b.wt.). Animals were sacrificed 16 h following TAA administration. $*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. The decrease in TAA associated rise in the enzyme activity is dependent upon the dose of AP. AP: allopurinol; TAA: thioacetamide; NC: normal control.

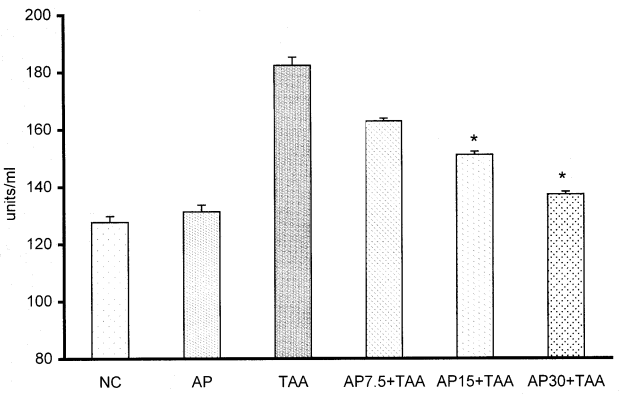

Fig. 2. sASP activity level in rats treated with AP followed by a necrogenic dose of TAA (400 mg/kg b.wt.). The value of AP alone (AP_{30}) shown in the figure is the value obtained from the group of rats given a dose of 30 mg/kg b.wt. for three consecutive days. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of TAA. Animals were sacrificed 16 h following TAA administration. $*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. The decrease in TAA associated rise in the enzyme activity is dependent upon the dose of AP. AP: allopurinol; TAA: thioacetamide; NC: normal control.

the cytotoxic effect of XO-derived ROM, generated in excess after TAA exposure.

3.2. Alleviation by AP of TAA-induced rise of serum markers of hepatic lesions (necrosis)

Activity levels of sALT, sASP and sALP were measured in rats given various doses of AP followed by TAA. Dose-dependent reductions in the activity levels of these biochemical parameters associated with necrotic lesions are shown in Figs. 1–3. AP pretreatment could significantly control the TAA-induced rise in the activity level of serum transaminases (aminotransferases) and sALP. The values were almost brought down to normal in the rats receiving 30 mg/kg b.wt. of AP (AP_{30}). Pretreatment of rats with AP is, therefore, observed to protect liver necrosis.

3.3. Hepatic malondialdehyde level and reduced GSH concentration in rats given AP followed by TAA

TAA administration promoted lipid peroxidation (as indicated by a rise in tissue malondialdehyde level, Table 2), and lowered the level of GSH in rat liver

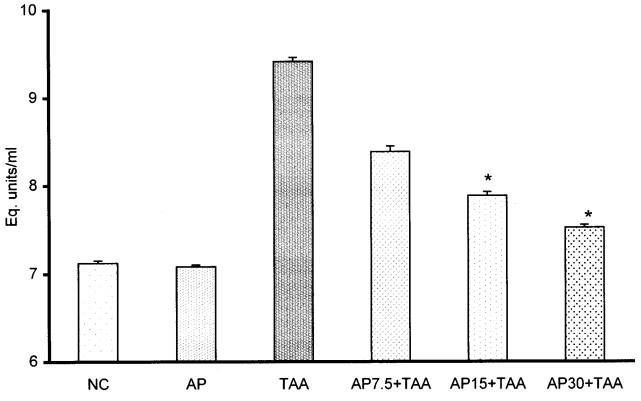

Fig. 3. Activity of sALP in rats treated with AP followed by a necrogenic dose of TAA. The value of AP alone (AP_{30}) shown in the figure is the value obtained from the group given a dose of 30 mg/kg b.wt. for three consecutive days. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of TAA (400 mg/kg b.wt.). Animals were sacrificed 16 h following TAA administration. $^*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. The decrease in TAA associated rise in the enzyme activity is dependent upon the dose of AP. AP: allopurinol; TAA: thioacetamide; NC: normal control.

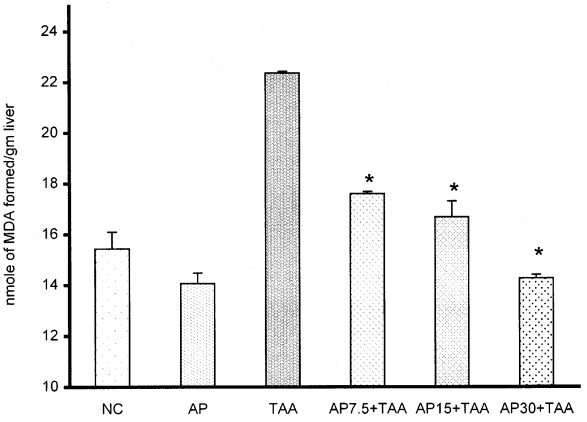

Fig. 4. Hepatic lipid peroxidation, measured as malondialdehyde content, in rats treated with AP followed by a single necrogenic dose of TAA. The value of AP alone (AP_{30}) shown in the figure is the value obtained from the group given a dose of 30 mg/kg b.wt. for three consecutive days. An increase in lipid peroxidation is observed in rats receiving TAA alone. Treatment with AP prior to TAA administration could significantly alleviate the levels of tissue malondialdehyde. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of TAA (400 mg/kg b.wt.). Animals were sacrificed 16 h following TAA administration. $^*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. AP: allopurinol; TAA: thioacetamide; NC: normal control; MDA: malondialdehyde.

(Table 2). Contrary to the effect of TAA treatment alone, pretreatment with AP followed by TAA could alleviate the TAA-induced rise in lipid peroxidation (Fig. 4) in a dose-dependent manner. This alleviation can be attributed to the ability of AP to inhibit XO, thereby inhibiting the generation of XO-derived ROM, which are proposed to lead to liver necrosis.

An increase in reduced GSH content of the hepatic tissue was also observed in the group of rats pretreated with AP. The effect of various doses of AP on GSH level is shown in Fig. 5. While the administration of TAA led to a marked decrease in GSH concentration, the pretreatment of rats with AP followed by TAA could cause a rise in hepatic GSH level. As shown in Fig. 5, the effect is dependent on the dose of AP.

3.4. Activity of hepatic XO in rats receiving AP before the administration of TAA

Fig. 6 illustrates the results of the effects of various doses of AP on TAA-induced rise in the activity of

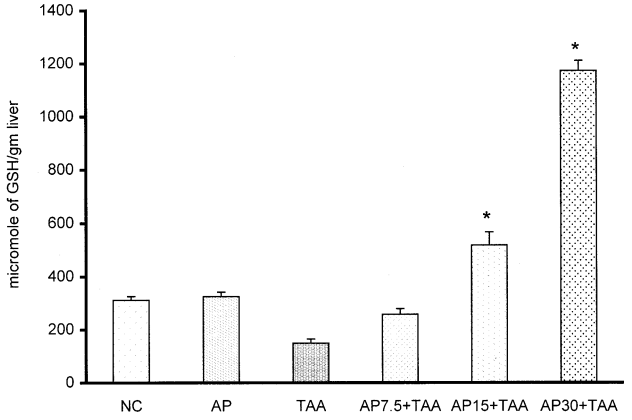

Fig. 5. Reduced GSH concentration in the liver tissue of rats pretreated with AP followed by a single necrogenic dose of TAA. Animals were sacrificed 16 h following TAA administration. The value of AP alone (AP_{30}) shown in the figure is the value obtained from the group of rats receiving a dose of 30 mg/kg b.wt. for three consecutive days. Treatment with AP prior to TAA could markedly enhance the amount of reduced GSH in the tissue. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of TAA (400 mg/kg b.wt.). $*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. AP: allopurinol; TAA: thioacetamide; NC: normal control; GSH: reduced GSH.

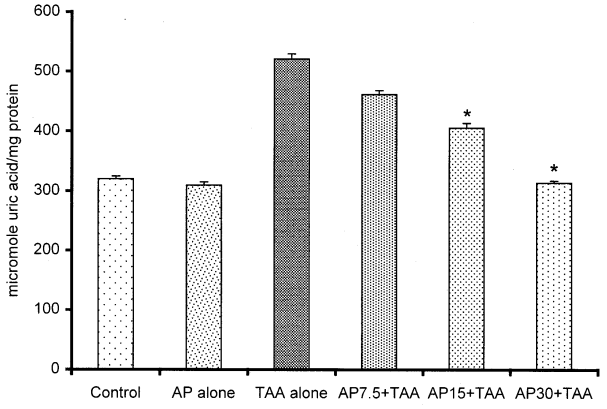

Fig. 6. Effect of AP pretreatment on TAA-induced rise in the activity of hepatic XO in rats. The value of AP alone (AP_{30}) shown in the figure is the value obtained from the group given a dose of 30 mg/kg b.wt. for three consecutive days. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days prior to the administration of a single necrogenic dose of TAA (400 mg/kg b.wt.). The animals were sacrificed 16 h after TAA administration. $*P < 0.05$ ($n = 6$), when the AP pretreated groups were compared with the TAA alone treated group. AP: allopurinol; TAA: thioacetamide.

XO in rat liver. Treatment with AP followed by TAA could inhibit the TAA-induced rise in XO activity. The effect is very much dependent on the dose of AP, and at a dose of 30 mg/kg b.wt., the enzyme activity was observed to be within the control limit. Further, decreased XO activity is clearly seen to be associated with a reduction in tissue malondialdehyde content (Fig. 6 versus Fig. 4), suggesting that the treatment with the inhibitor could alleviate the damage process, which involved ROM. The results shown in Figs. 1–4 correlate the inhibition of XO activity (consequently, of the generation of XO-derived ROM) with the suppression of necrosis as manifested by the reduction in the levels of biochemical parameters raised after TAA treatment.

3.5. *Effect of XO inhibitors on the TAA-induced lethality in rats*

The lethal dose of TAA killed nine out of 10 rats. Pretreatment of different groups of rats with various doses of AP ($AP_{7.5}$, AP_{15}, or AP_{30}) for three consec-

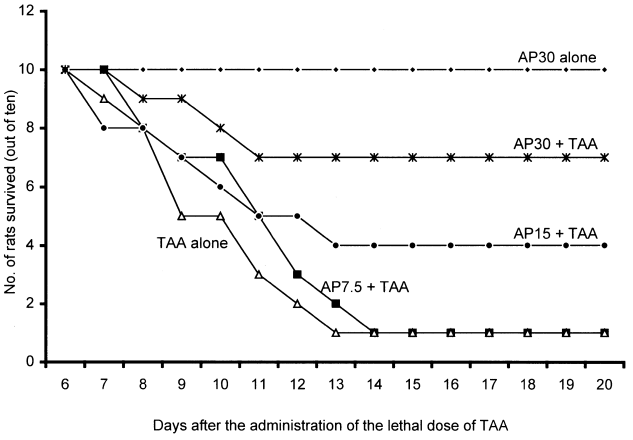

Fig. 7. Survival rate in rats receiving AP followed by the lethal dose of TAA. Rats were given AP for three consecutive days before administering the lethal dose of TAA. Dose and mode of administration of the hepatotoxin have been described in Section 2. The value of the AP alone treated group (AP_{30}) is the value obtained from the group given 30 mg AP/kg b.wt. for three consecutive days. $AP_{7.5}$, AP_{15} and AP_{30} indicate different doses of AP (viz. 7.5, 15, 30 mg/kg b.wt.) given for three consecutive days before the administration of the lethal dose of TAA. Survival was observed daily. Nine out of 10 rats died in the group receiving the lethal dose of TAA. AP pretreatment could increase the survival rate in rats receiving the lethal dose of TAA. AP: allopurinol; TAA: thioacetamide.

utive days prior to the administration of the lethal dose of TAA resulted in an appreciable decrease in mortality. The effect on survival, as shown in Fig. 7, is dependent on the dose of AP. The group of rats pretreated with a dose of AP_{30} exhibited a decrease in mortality up to 60% in comparison to the rats receiving the lethal dose of TAA alone. No mortality was observed in the group receiving AP alone. Tungsten, which is another inhibitor of XO, was also found to protect the rats against TAA-induced lethality (Fig. 8).

4. Discussion

A number of drugs, chemicals and also virus have been reported to cause liver necrosis, which sometimes becomes difficult to manage by medical therapies [14,15]. Effective protective agents may provide a choice for the patients at risk of hepatic failure due to severe necrosis. In the present study, we examined the potential of XO-derived ROM in producing necrosis. In vivo XO inhibitor AP is shown to be an effective anti-hepatotoxic agent when given prophylactically to rats.

Several studies have employed TAA to produce a model of hepatic necrosis in experimental animals [3,4,16]. The necrotic model induced by TAA in rats proved to be a reliable and satisfactory model [2]. The necrogenic dose of TAA administered to rats could cause a significant increase in sALT, sAST and sALP levels in rats sacrificed 16 h following TAA administration (Table 1). These results indicate liver necrosis and are consistent with the literature [2–4].

The production of hepatic injury by TAA is thought to be the result of binding of some of the TAA metabolites to cellular macromolecules [5–7]. In addition, radical reactions are suggested to be involved in the pathogenesis of liver injury caused by TAA [2]. To elucidate the possible role of ROM in the mechanism underlying TAA hepatotoxicity, the activity of rat liver XO was determined. Our data reveal that XO activity in rat liver is increased and, due to its ability to generate ROM, oxidative stress is produced, which is suggested to contribute to liver necrosis.

Structural and functional alterations associated with the exposure of the tissue to a wide variety of factors including many chemicals are understood to be mediated by ROM, which are generated at both the intercellular and intracellular level [17]. XO (EC 1.1.3.22) has been postulated as one of the primary intracellular sources of ROM and its activity level is reported to increase in several disorders including brain tumors [18]. It catalyzes the oxidation of hypoxanthine to xanthine and of the latter to uric acid. The enzyme derives from an NAD^+-dependent dehydrogenase by proteolysis [19] or by reversible oxidation of sulfhydryl groups [20]. While oxidizing its substrate, XO (in its oxygen-dependent form) generates partially reduced oxygen moieties such as superoxide anion radical and hydrogen peroxide as by-product, which can further be converted to a highly reactive hydroxyl radical by the Haber–Weiss and Fenton reaction [21]. Together, these partially reduced oxygen moieties are commonly referred to as ROM. The hyperactivity of XO would, therefore, lead to increased production of ROM.

ROM are generally detoxified by a battery of enzymatic and other non-enzymatic antioxidant defenses. An imbalance between the production and detoxification of ROM results in oxidative stress, which, through a number of processes including lipid peroxidation, lead to necrosis [22]. As can be seen from the results, administration of TAA to rats could cause an increase in the levels of biochemical markers of liver necrosis (Table 1) and also the activity of hepatic XO (Table 2). An increase in hepatic malondialdehyde content of the tissue, which is a marker of the oxidative damage provides evidence supporting the role of XO-derived ROM in the necrotic process by TAA (Fig. 4).

Reducing the oxidant production in cell by suppressing the radical reactions is one of the major protective mechanisms of the body under conditions of oxidative stress. Prophylactic treatment of rats with AP, an inhibitor of XO [23], is observed in this study to restore the normal levels of various biochemical parameters altered in the necrotic tissue (Figs. 1–4). As already discussed, ROM can perturb the structural integrity of the cell, for example, by causing the peroxidation of membrane lipids rich in unsaturated fatty acids, thereby resulting in the release of cytosolic enzymes from liver cells into the plasma. Treatment with AP seems to result in the reduced generation of ROM, thus preventing the

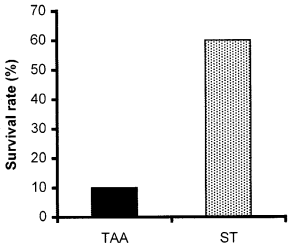

Fig. 8. Effect of tungsten pretreatment on TAA-induced lethality in rats. Dose and other details have been described in Section 2. TAA: thioacetamide; ST: sodium tungstate. A higher survival rate in tungsten pretreated group of rats can be seen.

damage, as is evident by the inhibition of the release of cytosolic enzymes from the cell. Changes in the hepatic malondialdehyde content prove in part that the anti-hepatotoxic effects of the pretreatment with AP are due to the inhibition of free radical formation. The effect of AP was so pronounced that it could cause an appreciable increase in survival rate in rats given a highly toxic dose of TAA (Fig. 7).

XO inhibitor AP may further help reduce the tissue injury by acting as direct free radical scavenger [23]. The protective effect of AP on TAA-induced mortality in rats, however, does not appear to be due to its antioxidant function. To clarify this point, tungsten was tested for its protective effect on TAA-induced lethality in rats. Tungsten is a XO inhibitor and, unlike AP, is not reported to act as a free radical scavenger [24]. When administered as ST to experimental animals, tungsten inhibits the activity of XO by slowly replacing molybdenum, the cofactor of the enzyme. As shown in Fig. 8, the survival studies clearly demonstrate that tungsten pretreatment can protect the rats against the life-threatening TAA hepatotoxicity, indicating that the liver necrosis is contributed to by ROM produced by XO, which is significantly induced in TAA treated rats. It, therefore, can be said that xanthine–XO system plays an important role in TAA hepatotoxicity.

To mitigate and repair the damage initiated by ROM, cells have evolved complex antioxidant systems, which help in detoxification. Earlier hypotheses cited to understand the oxidative damage by various chemical compounds suggest that oxidative stress will also occur if the detoxification mechanisms are impaired. The level of reduced GSH, which constitutes one of the physiologically important protective mechanisms to curtail progression of tissue damage [25] is generally affected under conditions of oxidative stress. Profound depletion of GSH may lead to necrosis, as its unavailability will make the tissue susceptible to damage by free radicals, which are produced in excess under stress conditions. During the present investigation, we observed a decrease in GSH content of the hepatic tissue in TAA treated rats (Table 2).

Depletion of GSH in the liver after the injection of TAA supports the view that radical reactions take place extensively in the liver during necrosis. Administration of AP prior to TAA is observed to result in a dose-dependent increase in the hepatocyte GSH level (Fig. 5), confirming that the damage is oxidative. The tissue GSH content, however, increased largely in the AP_{30}+TAA group more than that in the control group. Reduced GSH is a unique cellular protective agent which acts as both a nucleophilic scavenger of numerous compounds and their metabolites, converting electrophilic centers to thioether bonds, and as a cofactor in the GSH peroxidase-mediated destruction of hydroperoxides. Studies with chemicals like acetaminophen [26] and bromobenzene [27] have very clearly demonstrated that bioactivation of these chemicals followed by GSH adduct formation causes depletion of cytosolic GSH and oxidative stress. TAA, a substrate for the flavin-dependent monooxygenase present in the microsomes [28], is converted to toxic metabolites, which may be conjugated with GSH to facilitate their elimination. This excess demand of GSH may initiate the cellular response that causes an increase in the de novo synthesis of GSH, which is also required to counteract the cytotoxic effects of XO-derived ROM. Following the inhibition of XO, the production of ROM was also reduced and, therefore, much of the GSH pool of the tissue remained unused. This may provide an explanation for the large increase in hepatic GSH content in the group of rats pretreated with AP followed by TAA.

Analysis of the results presented in this paper clearly points out that there is an overall increase in the activity of liver XO, hepatic malondialdehyde content and the serum enzymes associated with liver necrosis. Hepatocyte GSH (reduced form) content was, as expected, lowered in rats receiving TAA. The results suggest that oxidative stress plays a crucial role in the necrotic process induced by TAA.

Pretreatment with AP followed by TAA is observed to attenuate the pro-oxidant effect as well as to normalize the toxin-mediated rise in aminotransferases and alkaline phosphatase activity levels. The protection against necrosis by AP seems to be the result of the inhibition of XO-catalyzed generation of ROM. Suppression of ROM generation by XO inhibitors has previously been shown to ameliorate the oxidation of biomolecules in other systems [18]. An increase in the survival rates in rats given the enzyme inhibitor prior to the administration of the lethal dose of TAA further strengthens the proposal that an increase in XO activity is a contributing factor in TAA-induced liver necrosis.

The mechanism by which XO is induced in the TAA treated group of rats is not clear. A possible explanation of this may be the oxidation of xanthine dehydrogenase by TAA itself or by the by-products generated during TAA metabolism. Xanthine dehydrogenase has been reported to undergo reversible oxidative alteration at its sulfhydryl group to form XO in vitro [19]. Conversion of xanthine dehydrogenase to XO might be a consequence of the intermediates generated as a result of primary or secondary oxidative reactions initiated by TAA within the biological system.

In summary, in these studies the administration of the hepatotoxic compound TAA is reported to cause an increase in the activity of rat liver XO, which through the generation of ROM can damage the tissue. The hepatic injury can be prevented by the administration of enzyme inhibitor AP in a dose-dependent manner. Inhibition of the enzyme activity and the concomitant reduction of oxidative stress, as manifested by a decrease in hepatic lipid peroxidation in rats pretreated with AP led us to suggest that the oxidant by-products generated during XO-catalyzed reaction play a crucial role in liver necrosis and that suppressing the XO-catalyzed generation of ROM can protect the tissue against necrosis. Further studies would be necessary to determine whether these results may have therapeutic applications in the future.

Acknowledgements

Mr. Siraj Hussain (IAS), Vice Chancellor, Jamia Hamdard is acknowledged for his interest and support. The authors acknowledge the CSIR for providing a fellowship to S.P.

References

[1] W.R. Lee, Acute liver failure, N. Engl. J. Med. 329 (1993) 1862–1872.
[2] R. Bruck, H. Aeed, H. Shirin, Z. Matas, L. Zaidel, Y. Anvi, Z. Halpern, The hydroxyl radical scavengers dimethylsulfoxide and dimethylthiourea protect rats against thioacetamide-induced fulminant hepatic failure, J. Hepatol. 31 (1999) 27–38.
[3] F. Sun, S. Hayami, Y. Ogiri, S. Haruna, K. Tanaka, Y. Yamada, S. Tokumaru, S. Kojo, Evaluation of oxidative stress based on lipid hydroperoxide, vitamin C and vitamin E during apoptosis and necrosis caused by thioacetamide in rat liver, Biochim. Biophys. Acta 1500 (2000) 181–185.
[4] S. Ali, K.A. Ansari, M.A. Jafry, H. Kabeer, G. Diwakar, *Nardostachys jatamansi* protects against liver damage induced by thioacetamide in rats, J. Ethnopharmacol. 71 (2000) 359–363.
[5] A.L. Hunter, H.A. Holscher, R.A. Neal, Thioacetamide-induced hepatic necrosis. Involvement of mixed function oxidase enzyme system, J. Pharmacol. Exp. Ther. 200 (1977) 439–448.
[6] W.R. Porter, R.A. Neel, Metabolism of thioacetamide and thioacetamide *S*-oxide by rat liver microsomes, Drug Metab. Dispos. 6 (1978) 379–388.
[7] A. Zaragoza, D. Andres, D. Sarrion, M. Cascales, Potentiation of thioacetamide hepatotoxicity by phenobarbital pretreatment in rats. Inducibility of FAD monooxygenase system and age effect, Chem. Biol. Interact. 124 (2000) 87–101.
[8] S. Reitman, S. Frankel, A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases, Am. J. Clin. Pathol. 28 (1957) 56–63.
[9] O.A. Bessey, O.H. Lowry, M.J. Brock, A method for the rapid determination of alkaline phosphatase with five cubic millimeter of serum, J. Biol. Chem. 164 (1946) 321–328.
[10] F. Bernheim, M.L.C. Bernheim, K.M. Wilburn, The reaction between thiobarbituric acid and the oxidation products of certain lipids, J. Biol. Chem. 174 (1948) 257–264.
[11] D.J. Jollow, J.R. Mitchell, N. Zampaglione, J.R. Gillete, Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4-bromobenzene as the hepatotoxic intermediate, Pharmacology 11 (1974) 151–169.
[12] F. Stirpe, E. Della Corte, Spectrophotometric estimation of xanthine oxidase, J. Biol. Chem. 244 (1969) 3855–3863.
[13] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin-phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[14] J.M. Mehta, S.G. Karamarkar, B.D. Pimparkar, U.K. Sheth, Levodopa in the treatment of hepatic coma due to fulminant hepatic failure, J. Postgrad. Med. 22 (1976) 32–36.

[15] J. Bernuau, J.P. Benhamou, Fulminant and subfulminant liver failure, in: N. McIntyre, J.P. Benhamou, J. Bircher, M. Rizzeto, J. Rodes (Eds.), Oxford Textbook of Clinical Hepatology, Oxford University Press, Oxford, 1991, p. 942.
[16] A. Akbay, K. Cinar, O. Uzunalimoglu, S. Eranil, C. Yurdaydin, H. Bozkaya, M. Bozdayi, Serum cytotoxin and oxidant stress markers in *N*-acetylcysteine treated thioacetamide hepatotoxicity of rats, Hum. Exp. Toxicol. 18 (1999) 669–676.
[17] J. Feher, G. Csomos, A. Vereckei, Role of free radical reactions in liver diseases, in: G. Csomos, J. Feher (Eds.), Free Radicals and Liver, Springer, Berlin, 1992, pp. 1–12.
[18] W.S. Chang, Y.H. Chang, F.J. Lu, H.C. Chiang, Inhibitory effects of phenolics on xanthine oxidase, Anticancer Res. 14 (1994) 501–506.
[19] E. Della Corte, F. Stirpe, The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme, Biochem. J. 126 (1972) 739–745.
[20] M.G. Batelli, E. Lorenzoni, F. Stirpe, Milk oxidase type D (dehydrogenase) and type O (oxidase). Purification, interconversion and some properties, Biochem. J. 131 (1973) 191–198.
[21] C.C. Winterbourn, H.C. Sutton, Iron and xanthine oxidase catalyzes formation of an oxidant species distinguishable from $OH^{\bullet}$: comparison with the Haber–Weiss reaction, Arch. Biochem. Biophys. 244 (1986) 27–34.
[22] W.N. Aldridge, Mechanism of toxicity: new concepts are required in toxicology, Trends Pharmacol. Sci. 2 (1981) 228–231.
[23] R.C. Moorehouse, M. Grootveld, B. Halliwell, J. Quiinlan, J.M.C. Gutteridge, Allopurinol and oxypurinol are hydroxyl radical scavengers, FEBS Lett. 213 (1987) 23–28.
[24] J.L. Johnson, W.R. Waud, H.J. Cohen, K.V. Rajagopalan, Molecular basis of the biological function of molybdenum. Molybdenum-free xanthine oxidase from livers of tungsten-treated rats, J. Biol. Chem. 249 (1974) 5056–5061.
[25] S. Ali, M. Abdulla, M. Athar, L-2-Oxothiazolidine-4-carboxylate, an in situ inducer of glutathioine, protects against paraquat-mediated pulmonary damage in rat, Med. Sci. Res. 24 (1996) 699–701.
[26] J.A. Hinson, N.R. Pumford, D.W. Roberts, Mechanisms of acetaminophen toxicity: immunochemical detections of drug protein adducts, Drug Metab. Rev. 27 (1995) 73–92.
[27] J. Thor, P. Moldeus, R. Hermanson, J. Hogberg, D.J. Reed, S. Orrenius, Metabolic activation and hepatotoxicity. Toxicity of bromobenzene in hepatocytes isolated from phenobarbital-treated and diethylmaleate-treated rats, Arch. Biochem. Biophys. 188 (1978) 122–129.
[28] H.V. Vadi, R.A. Neal, Microsomal activation of thioacetamide *S*-oxide to a metabolite(s) that covalently binds to calf thymus DNA and other polynucleotides, Chem. Biol. Interact. 35 (1981) 25–38.