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Dissociation of xanthine oxidase induction and cytochrome P450 depression during interferon induction in the rat

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Abstract—Interferon (IFN) and IFN inducers down-regulate hepatic cytochrome P450 (P450) through a pretranslational mechanism involving depression of P450 mRNA levels and a subsequent decrease in P450 synthesis. Current evidence suggests that interferon induces the synthesis of a protein which subsequently mediates the down-regulation of P450. Xanthine oxidase (XO) activity is induced by interferons in rodents, and the XO inhibitor allopurinol (AP) inhibits the down-regulation of P450 by interferons in the mouse and hamster so it has been proposed as the putative intermediate protein. In studies undertaken in rats to further characterize the molecular basis of the protective effect of AP, we observed that AP (20 and 50 mg/kg) did not protect against down-regulation of P450 by the interferon inducer polyinosinic-polycytidylic acid (10 mg/kg). In fact, at 50 mg/kg AP had an additive effect on the depression of CYP2E1. Total XO induction in the rat was only 30–50% compared with 100–500% in mice and hamsters, and this induction was inhibited completely by AP. Therefore, XO does not mediate the down-regulation of hepatic cytochrome P450 by interferons in the rat.

Interferon (IFN*) and IFN inducers down-regulate hepatic cytochrome P450 (P450) in a number of experimental species and in humans [reviewed in Refs 1 and 2]. The down-regulation of P450 occurs at a pretranslational step as demonstrated by a decrease in P450 mRNAs [3, 4] preceding a decrease in synthesis of P450 proteins [5]. Based on the ability of protein synthesis inhibitors to prevent the down-regulation of P450 by recombinant IFN, it has been postulated that an intermediate protein mediates the effects of IFNs [6]. In addition to down-regulating P450, IFNs regulate a number of acute phase and anti-viral proteins [7]. Xanthine oxidase (XO) activity is induced by IFN and IFN inducers [8]. Ghezzi *et al.* [9] proposed XO as the mediator protein based on the ability of allopurinol (AP), an inhibitor of XO, and free redical scavengers to block the down-regulation of P450 by the potent IFN α/β inducer polyinosinic-polycytidylic acid (polyIC) in mice. Other studies provided substantial but inconclusive support for this hypothesis [10–13]. Mannering *et al.* [14] questioned the XO hypothesis when they observed that the loss of XO in tungstate-treated mice did not affect the depression of P450 by interferon. The recent evidence demonstrating that the effects of interferon are mediated through down-regulation of cytochrome P450 mRNA [1, 3, 4] suggests that if XO is involved in the mechanism of action of interferon it would probably be via the production of free radicals that destroy mRNA rather than protein, as P450 protein degradation is only marginally if at all enhanced by interferons [5]. Therefore, AP could be effective in protecting against the effects of interferon by inhibiting XO and protecting mRNA. If XO does not mediate the effects of interferon on P450, AP-mediated protection of P450 would be through an XO-independent mechanism and its elucidation might aid in understanding the molecular mechanism by which interferons depress P450. The following experiments were undertaken to test these hypotheses concerning the role of AP and XO in IFN-mediated regulation of P450.

Materials and Methods

Materials. All reagents and chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) except as noted below.

Animals and treatment. Male Sprague–Dawley rats (200–250 g) obtained from Charles River were housed on clay chips and allowed free access to Purina rat chow and water *ad lib.* Rats were allowed to acclimatize in our facilities for 5 days before use. Rats were treated with either saline of polyIC (10 mg/kg) intraperitoneally at t = 0. Rats were gavaged with allopurinol (20 or 50 mg/kg) or saline 1 hr before and 6 hr after receiving polyIC or saline. Each treatment group (saline/saline; saline/allopurinol; polyIC/saline; polyIC/allopurinol) contained five animals. Two independent experiments with the two different doses of allopurinol were performed. Rats were killed at t = 24 hr by cervical dislocation, and livers were collected for the preparation of microsomes.

Methods. Hepatic microsomes were prepared from fresh liver by differential centrifugation of livers homogenized in ice-cold 1.15% KCl. Microsomes were resuspended in 1.15% KCl/100 mM sodium phosphate buffer, pH 7.4, and frozen at −70° until analyzed. Microsomal protein [15], P450 content [16], ethoxyresorufin *O*-deethylation (EROD) [17] and *p*-nitrophenol hydroxylation (PNPH) [18] were measured as previously described. Immunoquantitation of CYP2E1 was performed as previously described [18]: microsomal proteins were separated by electrophoresis on a 7.5% stacking gel under non-reducing conditions, transferred to a nitrocellulose membrane, probed with a form specific polyclonal rabbit anti-rat CYP2E1 (OXYgene Dallas, Dallas, TX, U.S.A.), and visualized via an alkaline phosphatase-labeled secondary antibody with nitro blue tetrazolium as a substrate.

XO activity was measured in the cytosolic fraction obtained after the preparation of the hepatic microsomes. Type O (xanthine oxidase; uses O_2 as an electron acceptor) and Type D (xanthine dehydrogenase; uses NAD^+ as an electron acceptor) xanthine oxidase activities were measured in the presence and absence of dithiothreitol (DTT) as described by Della Corte and Stirpe [19]. The sum of Type O and Type D xanthine oxidase activities measured in the presence of DTT reflects total xanthine oxidase activity.

Results were analysed by analysis of variance using Statview™ (Abacus Concepts, Berkeley, CA, U.S.A.) and Fisher's protected least squares difference for comparisons of means with a significance level of $P < 0.05$.

Results and discussion

These studies were undertaken with the objective of

* Abbreviations: IFN, interferon; P450, cytochrome P450; XO, xanthine oxidase; polyIC, polyinosinic-polycytidylic acid; AP, allopurinol; EROD, ethoxyresorufin *O*-deethylation; PNPH, *p*-nitrophenol hydroxylation; and DTT, dithiothreitol.

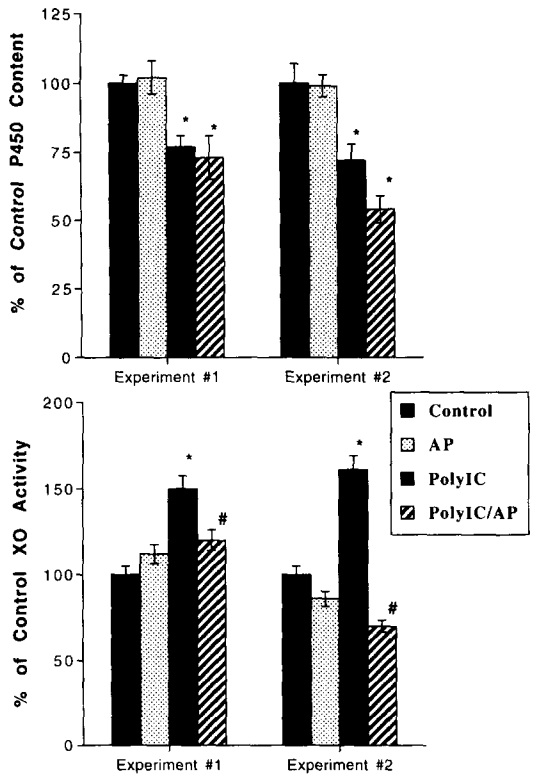

Fig. 1. Effect of allopurinol on polyIC-mediated down-regulation of P450 and induction of total XO activity. The effects of 20 mg/kg (experiment 1) and 50 mg/kg (experiment 2) AP on P450 content (top) and XO activity (bottom) and their response to polyIC administration are shown. Results are means ± SEM of N = 5. Control values were 0.74 ± 0.02 and 0.64 ± 0.04 nmol P450/mg protein and 2.5 ± 0.2 and 2.3 ± 0.5 nmol NADH equivalents/min/mg protein (total XO activity) in experiments 1 and 2, respectively. Key: (*) significantly different from control, $P < 0.05$; and (#) significantly different from polyIC, $P < 0.05$.

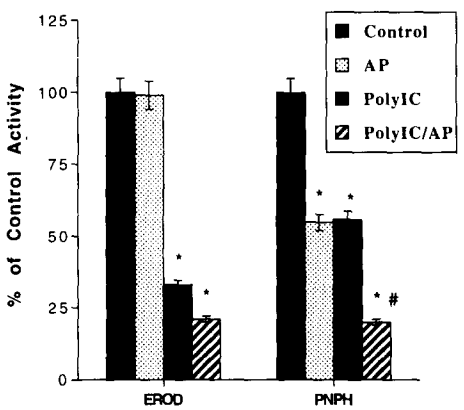

Fig. 2. Effect of 50 mg/kg on polyIC-mediated down-regulation of EROD and PNPH in hepatic microsomes. Results are means ± SEM of N = 5. Control values were 31 ± 2 and 540 ± 50 pmol/min/mg protein for EROD and PNPH, respectively. Key: (*) significantly different from control, $P < 0.05$; and (#) significantly different from AP and polyIC alone, $P < 0.05$.

elucidating the potential role of XO in mediating the down-regulation of P450 by the interferon inducer P450 and the mechanism by which AP appeared to protect against the down-regulation. As most of the mechanistic studies on down-regulation of P450 by interferons have been performed in the rat, the rat was selected as the species for these studies. In contrast to previous reports in hamsters and mice [9, 13], AP did not protect against the down-regulation of total P450 by polyIC (Fig. 1). This was observed despite the fact there was significant induction of total XO activity by polyIC, and this induction was inhibited completely by both dosage levels of AP (Fig. 1). The down-regulation of two P450-mediated oxidations followed that of total P450 (Fig. 2): PNPH is mediated predominantly

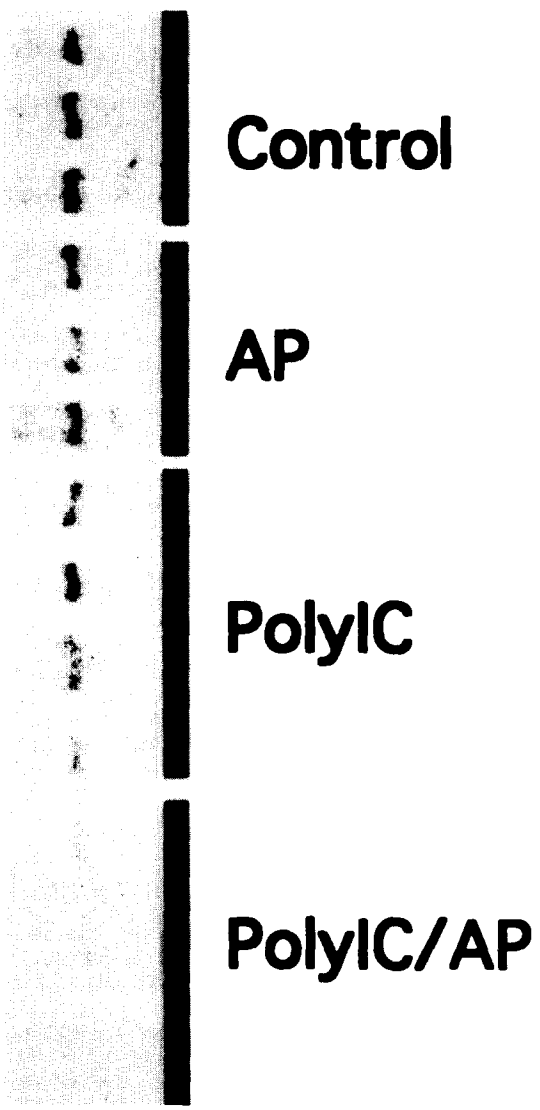

Fig. 3. Effect of 50 mg/kg AP on polyIC-mediated down-regulation of CYP2E1 protein as determined by immunoblotting (see Methods for details).

by CYP2E1 [18], whereas EROD is mediated primarily by CYP1A2 and to some extent by CYP2C11 in microsomes prepared from uninduced animals [20]. AP not only did not protect against down-regulation of PNPH but directly depressed this activity and had an additive effect with polyIC. Western blot analysis confirmed that CYP2E1 protein levels were affected in a manner paralleling the observed PNPH (Fig. 3). Western blot of CYP2C11 also showed a lack of protection at the protein level by AP (not shown).

In mice and hamsters, the increase in total XO activity by IFN and by polyIC tends to be much greater than that observed in rats. In the rat, increases of 30–60% have been reported [11], similar to those observed in this study, whereas in the mouse and hamster increases of 100–500% are usually observed [8, 10, 13]. It is possible, therefore, that XO may play a secondary role in enhancing P450 loss in some species by increasing degradation of P450 [21]. Despite essentially complete inhibition of XO induction in mice and hamsters, the protection afforded by AP is not complete [9, 13], which would not be inconsistent with a partial contribution by XO to the loss of P450. This would not appear to be the case in rats. In reviewing previous studies in mice, we noted that there was no correlation between the magnitude of XO induction and P450 loss [9, 10, 14] and that the induction of XO did not precede the depression of P450 but was coincidental [14]. Whether this reflects the independence of the two events or merely that the effect of XO induction on P450 had reached a maximum cannot be discerned from the literature. Interpretation of some studies [9] is also confounded by the fact that the doses of AP employed caused a direct depression of total P450 content.

As has been reported previously [10, 13, 14], the induction of total XO activity in our experiments was largely attributable to an increase in the convertible Type O form (reduced to Type D in the presence of DTT [19, 22]) in experiment 1 and to an increase in the Type D form in experiment 2 (data not shown). In neither experiment was there an increase in the non-convertible Type O form, which appears to arise through a proteolytic mechanism [22]. The normal physiological form of hepatic XO is Type D [22], and while it can lead to the formation of activated oxygen species, Type O is much more active in this regard [22]. It is unknown whether convertible Type O can exist *in vivo* [14, 22], and therefore total XO activity is the best reflection of the comparative potential to form activated oxygen species under these circumstances but is not a direct measure of the ability to do so.

The fact that no inhibition of total XO activity by AP was observed in control animals in our study probably relates to the mechanism of action of AP. AP is metabolised by the Type O form of XO to its active metabolite, oxipurinol, which subsequently binds to the XO reduced in the process (i.e. suicide inactivation) [23]. In contrast to other tissues, there is essentially no Type O in the liver [20] so that the suicide inactivation may not readily occur. Thus, when coupled with the dialysis step in the preparation of the cytosol fraction, inhibition of Type D XO would not be observed *in vitro*, while any Type O formed *in vivo* would be inactivated by the conversion of allopurinol to oxipurinol. Thus, for example in experiment 2, non-convertible Type O activity in control and polyIC-treated animals was inhibited by AP (45 and 75%, respectively), and the induced total XO activity in polyIC animals was inhibited totally.

Our results demonstrated that XO does not mediate P450 down-regulation by IFN in the rat and support the conclusion of Mannering *et al.* [14] that XO is not the primary mediator in mice. While the mechanism by which AP may provide partial protection in mice has yet to be resolved, this does not appear to be mechanistically relevant for future studies of the primary mechanism of action of IFN in P450 down-reguation in the rat because AP did not protect in that species. Future studies should rather be directed at identifiying the mechanism of mRNA down-regulation (mRNA stability vs transcriptional inhibition) and subsequent identification of putative ribonucleases or transcription factors that may be the intermediate protein.

Department of Pharmacology
Dalhousie University
Halifax, Nova Scotia
Canada, B3H 4H7

ALASTAIR E. CRIBB*
KENNETH W. RENTON†

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* Current address: Exploratory Biochemical Toxicology, Merck Research Laboratories, West Point, PA 19486.
† Corresponding author. Tel. (902) 494-3435; FAX (902) 494-1388.

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