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Requirement of cellular uptake for adenosine inhibition of *p*-nitrophenol glucuronidation in isolated rat hepatocytes

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Abstract—Adenosine (ADO) has been shown previously to inhibit *p*-nitrophenol glucuronidation in a concentration-dependent manner when concurrently incubated in isolated rat hepatocytes for 30–60 min. In the current study, preincubation of ADO (500 μM) in isolated hepatocytes for 30 min prior to addition of 100 μM *p*-nitrophenol resulted in a greater inhibition of glucuronidation when compared to that without preincubation (80 vs 50% inhibition). The inhibitory effect of 250 μM ADO on glucuronidation was decreased from 60 to 10% in the presence of the ADO transport inhibitor nitrobenzyl thioinosine during the 30-min preincubation period. Without prior incubation, 100 μM dibutyryl cyclic AMP (DBcAMP) produced an inhibition of glucuronidation similar to that of 500 μM ADO. In contrast to ADO, there was no significant difference in the inhibitory effect of DBcAMP on *p*-nitrophenol glucuronidation with or without a 30-min preincubation. Thus, DBcAMP and ADO appear to inhibit glucuronidation through different mechanisms. Furthermore, these results indicate that the inhibitory effect of ADO on *p*-nitrophenol glucuronidation is dependent to a large degree on the cellular uptake of ADO into hepatocytes, while a portion of the inhibitory effect may arise from the generation of intracellular cyclic AMP.

The purine nucleoside adenosine (ADO)* is an ubiquitous biologic compound which exerts a wide spectrum of effects on various tissues and organs throughout the body when released into the extracellular fluid under physiologic and pathophysiologic conditions [1, 2]. Many of these effects, which include cardiovascular, neuronal, renal and respiratory, depend on the interaction of ADO with a peripheral membrane-bound receptor [2–4]. However, previous studies have demonstrated that several effects of ADO arise from its uptake into the cell [5, 6]. This laboratory has shown previously that ADO inhibits the glucuronidation of *p*-nitrophenol in a concentration-dependent manner in isolated rat hepatocytes [7]. This inhibition was not caused by a decrease in UDP-glucuronosyltransferase (EC 2.4.1.17) activity, but was apparently the result of a decreased synthesis of UDP-glucuronic acid [7]. The mechanism of this inhibition is not understood; however, it appears that the effect is not solely the result of an ADO interaction with a hepatic membrane-bound receptor. Thus, the present study was designed to further elucidate the mechanism of action of ADO on *p*-nitrophenol glucuronidation in isolated rat hepatocytes by attempting to influence the inhibition with an ADO transport inhibitor, nitrobenzyl thioinosine (NBTI) [8].

Materials and Methods

Male Sprague–Dawley rats, weighing 200–350 g, were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). Animals were maintained in a temperature, humidity, and light-controlled room and allowed free access to water and Purina Rodent Laboratory Chow. Animals were fasted for 18–20 hr prior to being killed. Hepatocytes were isolated by a modification [9] of the procedure of Eacho and Weiner [10]. Metabolism was examined by incubating hepatocytes (2×10^6 cells/mL) in round-bottom flasks at 37° in a metabolic shaking bath (100 oscillations/min) under an atmosphere of 95% O_2/5% CO_2. Hepatocyte viability was determined using trypan blue exclusion, and only preparations with a viability of greater than 90% were used. Cells were allowed to equilibrate at 37° for approximately 2–3 min. ADO was then added to incubations in a small volume of Krebs–Ringer buffer. Following a second 2–3 min equilibration period, reactions were initiated by addition of *p*-nitrophenol. In a comparative set of experiments, hepatocytes were allowed to incubate with ADO for 30 min prior to the addition of *p*-nitrophenol. In some of the latter incubations, the ADO uptake inhibitor NBTI was added 5 min before the addition of ADO. At various incubation times, 4-mL aliquots of cell suspension were withdrawn and precipitated with 0.37 mL of 40% (w/v) trichloroacetic acid. Similar experiments were performed with dibutyryl cyclic adenosine 3′:5′-monophosphate (DBcAMP) replacing ADO. Control incubations, performed for each experiment, consisted of preincubation with buffer prior to addition of substrate. Viability checks were performed at the end of each incubation.

After acidification, precipitates were sedimented by centrifugation. The supernatant fluid obtained after centrifugation was adjusted to pH 5.0 with 2 N sodium acetate, and duplicate 1-mL samples were combined with 40 μL of β-glucuronidase (200 units). Additional duplicate 1-mL samples were used without enzyme addition to determine the level of unconjugated phenol. Samples without enzyme were alkalinized with 1.4 mL of 1.2 N sodium carbonate, and the absorbance was measured at 400 nm spectrophotometrically. Samples containing enzymes were covered and incubated at 37° for 2 hr in a shaking water bath (40 oscillations/min). At the end of the incubation period, the samples were alkalinized and read colorimetrically as was done with unconjugated phenol. Calibration curves were generated in the presence of β-glucuronidase to account for its absorbance. The quantity of conjugated metabolite was determined by subtracting the amount of free phenol detected in the absence of hydrolytic enzyme from the values obtained in samples that had undergone hydrolysis. Statistical analyses were performed utilizing Student's *t*-test.

ADO, DBcAMP, UDP-glucuronic acid, β-glucuronidase (Glucurase), and NBTI were obtained from the Sigma Chemical Co. (St. Louis, MO). Collagenase (type II) was purchased from Worthington (Freehold, NJ). *p*-Nitrophenol was obtained from the Eastman Chemical Co. (Rochester, NY). All other chemicals were of the highest reagent grade commercially available.

* Abbreviations: ADO, adenosine; cAMP, cyclic adenosine 3′:5′-monophosphate; DBcAMP, dibutyryl cyclic adenosine 3′:5′-monophosphate; and NBTI, nitrobenzyl thioinosine.

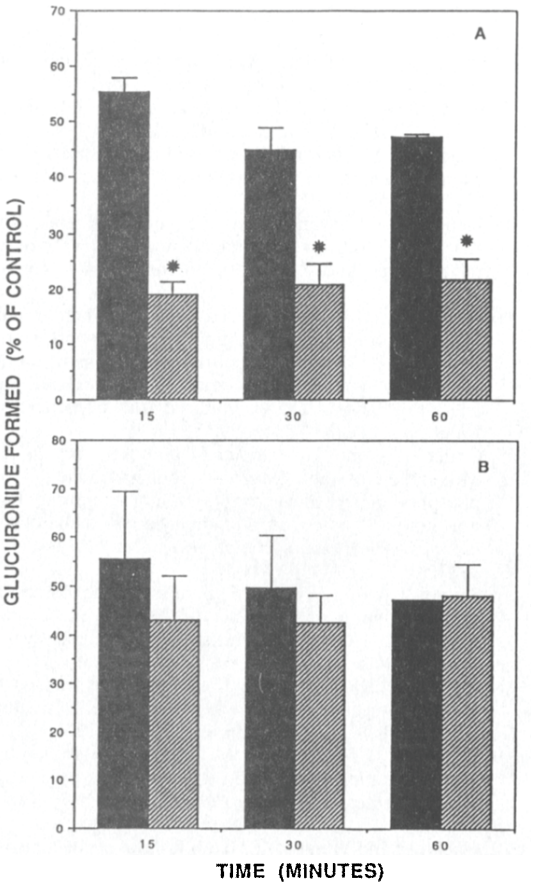

Fig. 1. Effects of preincubation time on inhibition of *p*-nitrophenol glucuronidation by ADO (A) and DBcAMP (B) in isolated rat hepatocytes. Hepatocytes were preincubated with 500 μM ADO or 100 μM DBcAMP either 2–3 min (■) or 30 min (▨) prior to addition of 100 μM *p*-nitrophenol and subsequent incubation for 15, 30 and 60 min. Values (means ± SEM) are expressed as percent of control cells obtained from 4 rats. Mean control values for *p*-nitrophenol glucuronidation obtained at 15, 30 and 60 min following 2–3-min or 30-min preincubations with buffer are listed in Results. Key: (*) significantly different from 2–3-min preincubation, $P < 0.05$.

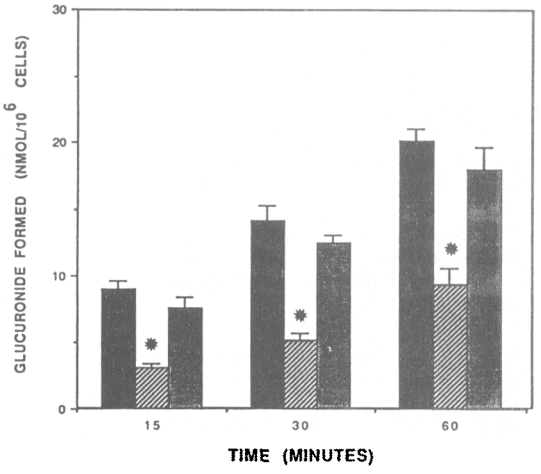

Fig. 2. Effects of NBTI on ADO-induced inhibition of *p*-nitrophenol glucuronidation in isolated rat hepatocytes. Hepatocytes were preincubated for 30 min with Krebs–Ringer buffer (■), ADO (250 μM) (▨), or ADO (250 μM) plus NBTI (0.5 μM) (□) prior to addition of 100 μM *p*-nitrophenol for 15, 30 and 60 min. Values represent means ± SEM of glucuronide formed/10^6 cells obtained from 4–6 rats. Key: (*) significantly different from both buffer (control) and ADO + NBTI values, $P < 0.05$.

Results

Addition of ADO (500 μM) 2–3 min prior to that of 100 μM *p*-nitrophenol inhibited glucuronidation of the latter compound by 44–55% when incubated in isolated rat hepatocytes from 15 to 60 min (Fig. 1A). The inhibitory effect of ADO on glucuronidation was further enhanced to approximately 80% by preincubation for 30 min prior to addition of substrate. The enhanced inhibition was similar when analyzed after 15-, 30- or 60-min incubations. Cell viability was not changed significantly after either experimental protocol.

As shown in Fig. 1B, incubation of hepatocytes with 100 μM DBcAMP caused an inhibition of glucuronidation similar to that of 500 μM ADO when substrate and inhibitor were incubated from 15 to 60 min. In contrast to results seen with ADO, preincubation for 30 min with DBcAMP did not cause further inhibition of *p*-nitrophenol glucuronidation. Control values (means ± SEM) for all 2–3-min preincubations were 7.20 ± 0.84, 14.12 ± 2.15 and 21.03 ± 2.30 nmol/10^6 cells measured at 15-, 30- and 60-min incubation times, respectively. Following all 30-min preincubations, control values (means ± SEM) were 9.78 ± 1.18, 15.78 ± 2.36 and 21.18 ± 2.57 nmol/10^6 cells measured at 15-, 30- and 60-min incubation times, respectively. Serving as controls for either ADO or DBcAMP experiments, preincubation with buffer produced glucuronidation values that were not significantly different from each other. Moreover, the 2–3- and 30-min preincubation control values were also similar to each other.

In the presence of 0.5 μM NBTI, the dramatic inhibition of glucuronidation caused by ADO was not observed (Fig. 2). NBTI reversed the inhibitory effects of ADO to 80% of control at all three incubation time points studied. NBTI alone did not modify significantly *p*-nitrophenol glucuronidation at any time period tested (15, 30 and 60 min); average values in the presence of NBTI alone were within 5–10% of control incubations.

Discussion

The present study demonstrates that a prolonged preincubation (30 min) of isolated hepatocytes with ADO prior to addition of *p*-nitrophenol resulted in a significantly greater inhibition of glucuronidation than that produced when both compounds were added several minutes apart. Since cell viability remained the same under both conditions, the decreased glucuronidation rate was not due to increased cell death. Furthermore, the inhibitory effect of ADO was substantially reversed by prior addition of NBTI, an ADO cellular transport blocker. These results suggest that ADO needs to enter the hepatocyte prior to maximally inhibiting this conjugation reaction.

That ADO requires cellular uptake for its effects was demonstrated recently by Oetjen *et al.* [5] who used NBTI to block the effects of ADO on glucagon-stimulated glucogen breakdown. In these investigations, a 50%

decrease in glucagon-stimulated glucose production via glycogen breakdown was observed when 20 μM ADO was added to cultured rat hepatocytes. The effect of ADO was not inhibited by 8-phenyltheophylline, a $P_1(A_2)$ ADO receptor antagonist [11], but was blocked by 200 μM NBTI. Further support of the necessity for cellular uptake was provided when it was demonstrated that 50% of the extracellular concentration of ADO was decreased after a 30-min incubation [5].

Prior studies in this laboratory [7] demonstrated that ADO decreases the synthesis of UDP-glucuronic acid, an essential cofactor for glucuronidation reactions. A precursor to UDP-glucuronic acid, cytosolic glucose-6-phosphate, has been shown to be sub-compartmentalized in rat hepatocytes into two different, mutually non-accessible pools, one linked to glycogenolysis and the other to gluconeogenesis [12]. Moreover, Banhegyi and coworkers [13] demonstrated that it is glycogenolysis, and not gluconeogenesis, that is the source of UDP-glucuronic acid utilized for glucuronidation.

While we suggest that cellular uptake is necessary for most of the response observed with ADO and glucuronidation, a small role may still exist for the second messenger cAMP. DBcAMP was a more potent inhibitor of glucuronidation than ADO; however, its effects were not blocked by the uptake inhibitor. Marchand *et al.* [14] demonstrated that in isolated rat hepatocytes, ADO increases the concentrations of ATP, ADP and cAMP. These changes were accompanied by a decrease in glycogenolysis, glucose consumption, and lactate production. They concluded that the increase in intracellular cAMP levels could not totally explain the metabolic changes that were observed, and suggested that a direct action of ADO may be involved. We propose that a similar system may be operating with regard to ADO-glucuronidation interactions. Since ADO still produced some degree of inhibition following blockade of its cellular uptake,and since DBcAMP produced a significant effect on glucuronidation, it is plausible that a portion of the ADO effect results from its interaction with an extracellular receptor to generate intracellular cAMP. Taken together, these results suggest that ADO may be taken up into hepatocytes, where it generates an inhibitory signal which produces anti-glycogenolytic effects, decreases UDP-glucuronic acid levels, and thus inhibits glucuronidation.

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