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EFFECTS OF ADENOSINE ON GLUCURONIDATION AND URIDINE DIPHOSPHATE GLUCURONIC ACID (UDPGA) SYNTHESIS IN ISOLATED RAT HEPATOCYTES

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Abstract—Dibutyryl cyclic adenosine 3′:5′-monophosphate (DBcAMP) has been shown to inhibit glucuronidation of *p*-nitrophenol in a concentration-dependent manner in isolated rat hepatocytes. Adenosine (ADO) also decreased glucuronidation in a similar fashion. The effects of adenosine were examined on the variables controlling glucuronidation in intact cells. The addition of adenosine was without effect on either glucuronyltransferase or β-glucuronidase. Adenosine decreased uridine diphosphate glucuronic acid (UDPGA) levels by 62% and, subsequently, inhibited glucuronidation by 41% in isolated rat hepatocytes. Since the synthesis of UDPGA requires NAD^+ for the dehydrogenation of UDP-glucose, alterations in the redox state could account for the decrease in intracellular UDPGA levels. The effects of ADO (500 μM) on lactate and pyruvate content and redox state were examined in rat hepatocytes. ADO caused a 2.1-fold increase in lactate levels and a 2.65-fold increase in the [lactate]/[pyruvate] ratio. The NAD^+/NADP ratio, therefore, was decreased by 63% in the presence of ADO. Carbohydrate reserve also affects UDPGA levels; thus, graded concentrations of glucose (5.5, 25, and 50 mM) were added to cells incubated with ADO. At 5.5 mM glucose, ADO caused a 61% decrease in glucuronide formation, while at concentrations of 25 and 50 mM glucose, the inhibition was diminished by 53 and 47% respectively. ADO appears to have decreased the synthesis of UDPGA by decreasing the NAD^+/NADH ratio, thus inhibiting UDP-glucose dehydrogenase. Carbohydrate reserve also appears to be involved in the inhibition of glucuronidation mediated by ADO.

Glucuronidation is a major pathway in the biotransformation of both foreign and endogenous compounds to less toxic, more water-soluble products [1]. Conjugation with glucuronic acid is dependent on the activity of a group of enzymes, the glucuronyltransferases, and the availability of UDPGA‡, an essential cofactor for glucuronidation [2]. Changes in hormonal status, as well as many pathological conditions, have been shown to decrease the formation of UDPGA and, therefore, the formation of glucuronides. Such conditions include hyperthyroidism [3], starvation [4,5], and experimental diabetes [6–8]. Since all these conditions [9, 14] have in common an increase in tissue cAMP via stimulation of adenylate cyclase, cAMP may play a role in the alterations seen in glucuronidation under these conditions.

Only limited studies have examined the effects of cAMP on glucuronidation. Pretreatment of rats with dibutyryl cAMP or glucagon has been shown to enhance glucuronyltransferase activity in liver homogenates [15]. Conversely, the addition of dibutyryl cAMP, ATP, ADP and AMP to isolated rat hepatocytes inhibits the glucuronidation of *p*-nitrophenol in a concentration-dependent manner [16, 17]. Adenosine has also been shown to decrease glucuronidation in a similar fashion [17]. Furthermore, Londos and Wolff [18] have demonstrated that adenosine and adenosine analogs, N^6-phenylisopropyladenosine and 5′-*N*-ethylcarboxamideadenosine, activate adenylate cyclase in a liver plasma-membrane preparation. Similar results have been shown with isolated rat hepatocytes using adenosine, PIA, and 2-chloroadenosine [19]. Bartrons *et al.* [19] also demonstrated that ATP and AMP produced the same effects as adenosine in hepatocytes because both are rapidly converted to adenosine by a membrane 5′-nucleotidase. These results suggest that the inhibition by adenine nucleotides may have been mediated by adenosine.

At least five rate-controlling factors have been identified for glucuronidation in intact cells: substrate supply, UDPGA levels, NAD^+/NADH redox state, carbohydrate reserve, and the activity of the glucuronyltransferases. Thus, the present studies were designed to examine the effects of adenosine on

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‡ Abbreviations: UDPGA, uridine diphosphate glucuronic acid; cAMP, cyclic adenosine 3′:5′-monophosphate; DBcAMP, dibutyryl cyclic adenosine 3′:5′-monophosphate; pNP, *p*-nitrophenol; PIA, N^6-phenylisopropyladenosine; TCA, trichloroacetic acid; and PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

these variables controlling glucuronidation in order to elucidate the mechanism(s) responsible for the inhibition.

MATERIALS AND METHODS

Chemicals. UDPGA, 4-methylumbelliferone, β-glucuronidase (Glucurase), Brij 58 (polyoxyethylene 20-cetyl ether), and 4-methylumbelliferonyl-β,D-glucuronide were obtained from the Sigma Chemical Co. (St Louis, MO). Collagenase (type II) was purchased from Worthington (Freehold, NJ). 4-Nitrophenol was obtained from the Eastman Chemical Co. (Rochester, NY). An A-gent Clinical Chemistry Reagent-Glucose UV kit was purchased from Abbott Diagnostic (Chicago, IL). All other chemicals were reagent grade and obtained from commercial sources.

Animals. Male Sprague–Dawley rats, weighing 200–300 g, and male Hartley guinea pigs, weighing 300–400 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 or Purina Guinea Pig Chow respectively. Unless otherwise stated, animals were fasted for 16–19 hr prior to being killed.

Tissue preparation and incubation conditions. Hepatic microsomes were prepared by differential centrifugation as previously described [20]. Hepatocytes were isolated by a modification [21] of the procedure of Eacho and Weiner [22]. Metabolism was examined by incubating hepatocytes (2×10^6 cells/ml) in round-bottom flasks at 37° in a Dubnoff 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. After a second 2–3 min equilibration period following the addition of adenosine in a small volume of Krebs–Ringer buffer, reactions were initiated by the addition of pNP. At various times, 6-ml aliquots of cell suspension were withdrawn and precipitated with 0.56 ml of 40% (w/v) TCA. Viability checks were performed at the end of each incubation.

The microsomal UDP-glucuronyltransferase assay was performed in a final volume of 0.5 ml in 17 × 100 mm polypropylene Falcon tubes. The reaction mixture contained 0.25 ml of microsomal suspension (1 mg protein), 0.1 ml of 0.2% Triton X-100 in 0.1 M phosphate buffer (pH 7.4), 2 mM UDPGA, 5.0 mM $MgCl_2$ and pNP as substrate. The reaction was initiated by addition of substrate and was carried out in a Dubnoff metabolic shaking bath (100 oscillations/min) at 37° for 15 min. After stopping the reaction with 1 ml of 10% (w/v) TCA, the precipitate was sedimented by centrifugation, and 1 ml of the supernatant fraction was used for analytical determinations.

Analytical determinations. 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) or arylsulfatase (5 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 on a Beckman model DU-8 spectrometer. Samples containing enzymes were covered and incubated at 37° in a shaking water bath (40 oscillations/min). Those samples containing β-glucuronidase were incubated for 2 hr and samples with arylsulfatase for 1 hr. At the end of the incubation periods, the samples were alkalinized and read colorimetrically as was done with the unconjugated phenol. Calibration curves were generated in the presence of each enzyme to account for their absorbance. The quantity of the specific conjugated metabolites was determined by subtracting the amount of free phenol detected in the absence of hydrolytic enzymes from the values obtained in samples that had undergone hydrolysis.

Arylsulfatase was dissolved in 0.2 N sodium acetate, pH 5.0; β-glucuronidase was purchased as a solution (Glucurase) in the same buffer. 1,4-Saccharic lactone, at a concentration of 20 mM, was incorporated into the arylsulfatase solution to inhibit β-glucuronidase activity which contaminates this preparation.

Microsomal UDP-glucuronyltransferase activity was determined by monitoring the disappearance of substrate, pNP. Absorbance of pNP was measured at 400 nm spectrophotometrically following addition of 1.4 ml of 1.2 N Na_2CO_3 to 1 ml of the TCA supernatant fraction.

UDPGA levels were determined enzymatically as previously described by Bock and White [23] as modified for hepatocytes by Ullrich and Bock [24]. Aliquots (2 ml) of cell suspension containing approximately 2×10^6 cells/ml were removed and boiled for 2 min. After heating, the suspensions were cooled on ice and centrifuged for 15 min at 10,000 *g*. UDPGA was determined in the supernatant fluid by means of the 4-methylumbelliferone-glucuronyltransferase assay [25].

Lactate was determined by the method of Noll [26] with modifications as described by Lowry and Passonneau [27]. The concentration of pyruvate was measured by the procedure of Kubowitz and Ott [28] as modified by Lowry and Passonneau [27]. The NAD^+/NADH ratio from lactate dehydrogenase was calculated as [pyruvate]/[lactate] × 1/*K*, where *K* = equilibrium constant of the enzyme [29].

Free glucose levels in hepatocytes were determined by a modification of the procedure of Slein [30] using an A-gent Clinical Chemistry Reagent-Glucose UV kit, a commercially available glucose kit (Abbott Laboratories). Glycogen content of hepatocytes was determined by the method of Keppler and Decker [31]. ATP, ADP, and AMP levels were determined by the method of Lowry and Passonneau [27].

Statistical analyses. Statistical analyses were performed utilizing Student's *t*-test.

RESULTS

After 30 min of incubation with 100μM pNP, pro-

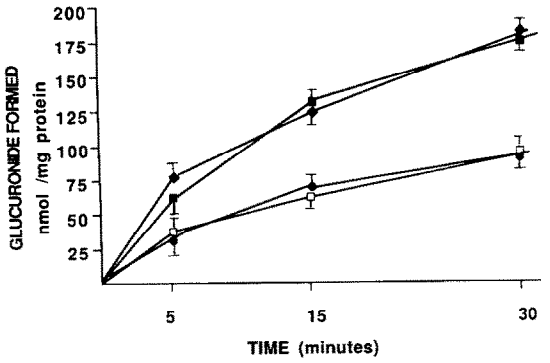

Fig. 1. Effects of adenosine on microsomal glucuronyltransferase activity. Microsomes were prepared as described in Materials and Methods. The amounts of pNP-glucuronide formed were determined at the indicated time intervals. Key: 100 μM pNP (□); 100 μM pNP plus 500 μM adenosine (●); 200 μM pNP (◆); and 200 μM pNP plus 500 μM adenosine (■). Each point represents the mean ± SEM for six animals.

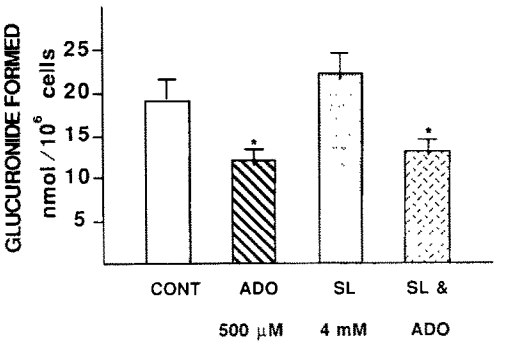

Fig. 2. Effects of 1,4-saccharic lactone on the inhibition of glucuronidation mediated by adenosine. Levels of pNP-glucuronide were determined in hepatocytes at 30 min. Each bar represents the mean ± SEM for at least four animals. Key: (*) significantly different from control values, $P < 0.05$.

duction of glucuronide conjugate was 23.83 ± 2.29 nmol/10^6 cells, while sulfate conjugate formation was 10.81 ± 1.92 nmol/10^6 cells in isolated hepatocytes from male fasted rats. The remainder of the 100 μM dose was accounted for by unmetabolized free pNP. Adenosine inhibited glucuronidation in a concentration-dependent manner (Table 1). Addition of adenosine (500 μM) to the incubation inhibited glucuronidation by 48% (Table 1) without affecting sulfation (data not shown). Since adenosine has been shown to increase cAMP levels in hepatocytes [19], the effects of cyclic nucleotides on glucuronidation were also examined (Table 1). Cyclic AMP did not alter glucuronidation significantly. However, both the dibutyryl and the 8-bromo derivatives of cAMP decreased glucuronide formation in hepatocytes.

Table 1. Effects of adenine nucleotides on glucuronidation of pNP in hepatocytes

Treatment	Concentration (μM)	Glucuronide formation (% of control)
Adenosine	500	52 ± 6*
	250	60 ± 4*
	100	75 ± 5
	50	88 ± 9
	5	104 ± 11
cAMP	1000	86 ± 9
	500	94 ± 9
DBcAMP	500	13 ± 2*
	200	45 ± 5*
	100	68 ± 6*
	50	76 ± 8
8-BrcAMP	500	29 ± 3*

Hepatocytes (30 ml) were incubated with nucleotides and 100 μM pNP for 30 min at 37° under an atmosphere of 95% O_2/5% CO_2. Glucuronidated metabolites were assayed as described in Materials and Methods. Control incubations were run for each experiment. The mean control value was 23.83 ± 2.29 nmol/10^6 cells. Values represent mean percent of control ± SEM obtained from hepatocytes of three to six fasted animals.

* Significantly different from control value, $P < 0.05$.

In an attempt to elucidate the mechanism(s) responsible for the inhibition of glucuronidation by adenosine, studies were performed to examine the effects of adenosine on parameters controlling glucuronidation. Net production of pNP-glucuronide could be diminished by either inhibition of glucuronyltransferase activity or increased β-glucuronidase activity. The inhibitory action of adenosine on pNP glucuronidation in hepatocytes was not caused by inhibition of glucuronyltransferase (Fig.1). It was found that microsomal glucuronyltransferase activity in the presence of 500 μM adenosine was not significantly different from control values at two substrate concentrations and three time periods. Nor was the ability of adenosine to decrease pNP glucuronide formation in intact cells by increasing breakdown of the glucuronide by β-glucuronidase (Fig. 2) responsible for the inhibition. When 1,4-saccharic lactone, an inhibitor of β-glucuronidase, was added to the incubations, it was unable to prevent the effects of adenosine on glucuronidation.

One of the major factors regulating glucuronidation in hepatocytes appears to be carbohydrate reserve; therefore, the effect of nutritional state on the inhibition of glucuronidation by adenosine was investigated (Fig. 3). The effects of adenosine and DBcAMP, alone and in combination, were examined in fed and fasted rats. The effects of all three treatments were attenuated in the fed animals as compared to the fasted rats. In fasted rats, adenosine, DBcAMP, and the two in combination caused a 34, 24, and 56% inhibition of glucuronidation, respectively, while in fed animals the inhibition was nonsignificant and averaged 12, 6, and 20% respectively.

Since glucuronic acid is derived from glycogen and glucose, the effects of adenosine on UDPGA levels and glucuronide formation were examined (Fig. 4). Adenosine (500 μM) decreased UDPGA levels by 62% and glucuronide formation by 41%. Similarly,

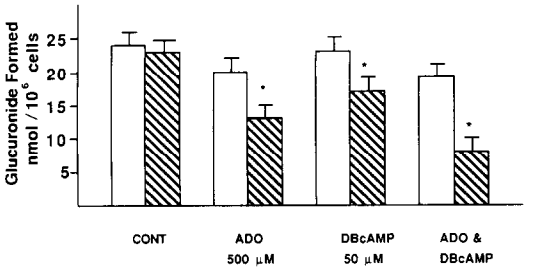

Fig. 3. Effects of feeding and fasting on the effects of adenosine, DBcAMP, and the two in combination on glucuronidation. Animals were fasted for 16–19 hr prior to being killed. Each bar represents the mean ± SEM of at least four animals. Key: (*) significantly different from corresponding fed rats at $P < 0.05$.

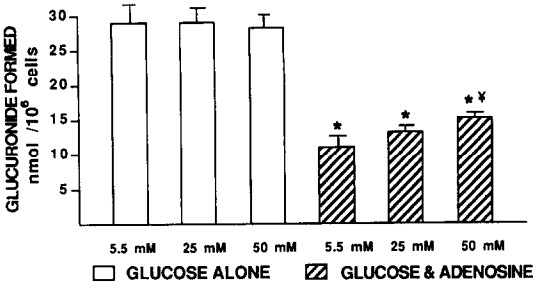

Fig. 5. Effect of additional glucose on the inhibition of glucuronidation by adenosine. Glucose was added to the hepatocyte preparations just prior to the beginning of the incubation period. Cells were incubated with 100 μM pNP for 30 min. Each bar represents the mean ± SEM for at least four animals. Key: (*) significantly different from control values, $P < 0.05$; (¥) significantly different from 5.5 mM glucose, $P < 0.05$.

the effects of DBcAMP on UDPGA levels and glucuronide formation were also studied. At all three concentrations of DBcAMP, UDPGA levels were diminished (Fig. 4). At 30 min, UDPGA levels were decreased by 82, 61, and 39% at 500, 100, and 50 μM DBcAMP, respectively, as compared to controls. In keeping with the decreases in UDPGA levels, glucuronide formation was also decreased (Fig. 4). At 30 min, glucuronide formation was inhibited by 88, 43, and 13% at 500, 100, and 50 μM DBcAMP respectively.

Further studies were performed to examine the effects of glucose concentration on the inhibition of glucuronidation in response to adenosine (Fig. 5). Cells were prepared in incubation buffer that contained physiological glucose concentrations (5.5 mM). At the beginning of the incubation period, graded concentrations of glucose were added. Alone, the additions of glucose were without effect on glucuronidation; however, in the presence of adenosine, the lower the concentration of glucose the greater the inhibition of glucuronidation by adenosine. At 5.5 mM glucose, adenosine caused a 61% decrease in glucuronide formation. At higher concentrations of glucose, 25 and 50 mM, the inhibition was diminished as 53 and 47% decreases were observed respectively. At the highest concentration of glucose (50 mM) added to the incubation, the level of inhibition of glucuronidation was significantly different from the inhibition at 5.5 mM glucose.

The ability of adenosine to alter the cellular content of glucose and glycogen was also examined (Table 2). Glycogen content was decreased significantly by 41% in cells incubated for 30 min with adenosine as compared to control cells. Glucose content tended to be decreased (10%). DBcAMP, at the two highest concentrations, caused significant decreases in glycogen content. At 500 and 100 μM, DBcAMP glycogen content was decreased by 49 and 38% respectively. Glucose content was also decreased significantly in cells incubated with 500 or 100 μM DBcAMP, 21 and 16% respectively. At 50 μM DBcAMP neither glycogen nor glucose content was altered significantly.

UDP-glucose dehydrogenase is an NAD^+-requiring enzyme responsible for UDPGA synthesis; therefore, changes in the redox state of NAD^+ could alter glucuronidation by modifying intracellular UDPGA levels. Lactate and pyruvate levels were determined in the presence and absence of adenosine (Table 3). Incubation with adenosine caused a 2.1-fold increase in lactate levels and a 2.65-fold increase

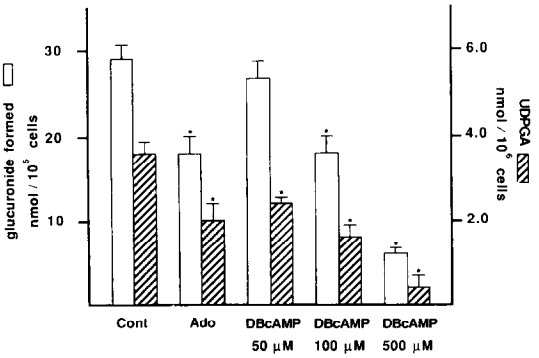

Fig. 4. Effects of adenosine and DBcAMP on UDPGA content and glucuronide formation. Cells were incubated with adenosine (500 μM) or DBcAMP for 30 min. Each bar represents the mean ± SEM of at least six animals. Key: (*) significantly different from control values, $P < 0.05$.

Table 2. Effects of adenine nucleotides on glucose and glycogen content of isolated hepatocytes

Treatment	Glucose (μmol/g)	Glycogen (μmol/g)
Control	3.60 ± 0.29	1.34 ± 0.09
Adenosine, 500 μM	3.34 ± 0.26	0.79 ± 0.15*
DBcAMP, 500 μM	3.04 ± 0.17*	0.68 ± 0.05*
DBcAMP, 100 μM	2.87 ± 0.11*	0.83 ± 0.10*
DBcAMP, 50 μM	3.19 ± 0.21	1.26 ± 0.12

Hepatocytes (30 ml) were incubated with adenine nucleotides and 100 μM pNP for 30 min at 37° under an atmosphere of 95% O_2/5% CO_2. Glucose and glycogen content were determined as described in Materials and Methods. The values are expressed as micromoles per gram of liver wet weight. Glycogen is expressed as glucose equivalents. Values are means ± SEM of three to four hepatocyte preparations.

* Significantly different from control, $P < 0.05$.

Table 3. Effects of adenine nucleotides on the redox state in isolated hepatocytes

Treatment	Pyruvate (μmol/g)	Lactate (μmol/g)	[Lact]/[pyr] ratio	NAD^+/NADH ratio
Control	0.38 ± 0.04	2.79 ± 0.57	7.44	1222
Adenosine, 500 μM	0.30 ± 0.05	5.88 ± 0.59*	19.53*	466*
DBcAMP, 500 μM	0.36 ± 0.09	2.94 ± 0.83	8.19	1109
DBcAMP, 100 μM	0.28 ± 0.05*	1.77 ± 0.45*	7.49	1218
DBcAMP, 50 μM	0.18 ± 0.09*	1.29 ± 0.44*	7.19	1264

Hepatocytes (30 ml) were incubated with nucleotides and 100 μM pNP for 30 min at 37° under an atmosphere of 95% O_2/5% CO_2. Lactate and pyruvate levels were determined as described in Materials and Methods. The NAD^+/NADH ratio from lactate dehydrogenase was calculated as [pyruvate]/[lactate] × 1/K, where K = equilibrium constant of the enzyme = 1.1×10^{-4} at 37° and pH 7.0 [27]. Values are expressed in micromoles per gram of liver wet weight. Values are means ± SEM of four experiments.

*Significantly different from control, $P < 0.05$.

in the [lactate]/[pyruvate] ratio. The NAD^+/NADH ratio was, therefore, decreased by 63% in the presence of adenosine. DBcAMP also caused changes in the lactate and pyruvate levels. Lactate levels were decreased by 36 and 54% by 100 and 50 μM DBcAMP respectively. Similarly, pyruvate content was decreased 26 and 53% by 100 and 50 μM DBcAMP; therefore, the [lactate]/[pyruvate] and NAD^+/NADH ratios remained unchanged.

The effects of adenosine on adenine nucleotide content (Table 4) and the ATP/ADP ratio were also investigated. At both 15- and 30-min incubations, adenosine caused significant increases in ATP, ADP, and AMP content and a total increase in nucleotide content of 42 and 79% at 15 and 30 min respectively. DBcAMP also caused significant increases in the adenine nucleotide content but only at the 30-min incubation (Table 4). Total adenine nucleotide content was increased by 50 and 38% by 500 and 100 μM DBcAMP respectively. However, only adenosine caused a significant increase in the ATP/ADP ratio. Adenosine increased the ATP/ADP ratio from 2.56 ± 0.01 in control cells to 3.19 ± 0.31 ($P < 0.05$). ATP/ADP ratios for cells incubated with DBcAMP were not significantly different from control cells; the ratios ranged from 2.44 ± 0.20 to 2.82 ± 0.16 (data not shown).

DISCUSSION

Several possible explanations for the inhibition of *p*-nitrophenyl glucuronide formation by adenosine in isolated hepatocytes have been evaluated. Among these are effects on enzyme activity, changes in nutritional state, alterations in cofactor supply, and disruption of the redox or energy state. Microsomal glucuronyltransferase activity is not altered by adenosine, nor is it changed by DBcAMP [32]. Thus, the inhibition of glucuronidation by adenosine cannot be attributed to decreases in glucuronyltransferase activity. This conclusion is further supported by the reported weak stimulation of glucuronyltransferase by adenine nucleotides [33, 34].

The possibility that the decrease in glucuronide

Table 4. Effects of adenosine and dibutyryl cAMP on adenine nucleotide content in hepatocytes

Treatment	Incubation time (min)	ATP (μmol/g)	ADP (μmol/g)	AMP (μmol/g)	Total change (%)
Control	0	1.17 ± 0.11	0.43 ± 0.09	0.14 ± 0.02	
	15	1.22 ± 0.13	0.44 ± 0.17	0.15 ± 0.03	
	30	1.23 ± 0.13	0.48 ± 0.10	0.15 ± 0.02	
Adenosine, 500 μM	0	1.22 ± 0.09	0.51 ± 0.09	0.11 ± 0.04	5
	15	1.70 ± 0.20*	0.55 ± 0.07*	0.32 ± 0.04*	42*
	30	2.27 ± 0.13*	0.71 ± 0.17*	0.35 ± 0.02*	79*
DBcAMP, 500 μM	0	1.00 ± 0.09	0.41 ± 0.12	0.12 ± 0.04	−12
	15	1.40 ± 0.13	0.46 ± 0.09	0.20 ± 0.03	14
	30	1.83 ± 0.14*	0.65 ± 0.06*	0.31 ± 0.05*	50*
DBcAMP, 100 μM	0	0.92 ± 0.16	0.36 ± 0.10	0.09 ± 0.05	−26
	15	1.44 ± 0.20	0.62 ± 0.24	0.15 ± 0.09	22
	30	1.69 ± 0.15*	0.66 ± 0.13	0.21 ± 0.08	38*
DBcAMP, 50 μM	0	1.26 ± 0.09	0.45 ± 0.11	0.15 ± 0.04	9
	15	1.44 ± 0.11	0.50 ± 0.12	0.12 ± 0.03	14
	30	1.49 ± 0.13	0.61 ± 0.14	0.20 ± 0.06	23

Hepatocytes (30 ml) were incubated with adenine nucleotides and 100 μM pNP at 37° under an atmosphere of 95% O_2/5% CO_2. Nucleotide content was determined as described in Materials and Methods. Values are expressed in micromoles per gram of liver wet weight. Values are means ± SEM of four experiments.

* Significantly different from control, $P < 0.05$.

formation was due to enhanced breakdown or deconjugation was also examined. Belinsky *et al.* [35] have demonstrated that epinephrine via α-adrenergic effects increases intracellular calcium which activates microsomal β-glucuronidase, causing a decrease in the net amount of glucuronide produced in the perfused rat liver. Recent studies have suggested that adenosine can increase calcium influx in the CNS through a receptor-mediated phenomenon [36–38]. Therefore, if adenosine were increasing intracellular calcium levels in the liver, the inhibition of glucuronidation caused by adenosine would be due to enhanced deconjugation via activation of the β-glucuronidase, not decreased formation of the glucuronide metabolite. This, however, does not appear to be the case. Incubation of cells with 1,4-saccharic lactone (4 mM), an inhibitor of β-glucuronidase [35, 39] did not alter the effects of adenosine on glucuronidation (Fig. 2).

Since glucuronic acid and, therefore, UDPGA are derived from glycogen and glucose, nutritional state and cofactor supply are related. When the effects of adenosine and DBcAMP, alone and in combination, were examined in fed and fasted rats, the inhibition of glucuronidation was attenuated in the fed animals. This greater inhibition in fasted rats suggests that carbohydrate reserve and thus cofactor supply were involved in the effects of adenosine and DBcAMP on glucuronidation. Since the rate of glucuronidation has been shown to be dependent on UDPGA levels in hepatocytes [40], subsequent studies have established that the inhibition of glucuronidation by adenosine is due to alterations in the cellular content of UDPGA. Adenosine, as well as DBcAMP, markedly decreased UDPGA content in isolated cells after a 30-min incubation. As with other compounds that deplete UDPGA levels in hepatocytes [40, 41], glucuronide formation was subsequently diminished.

Several studies have demonstrated that adenosine inhibits gluconeogenesis from lactate in isolated rat hepatocytes [19, 42, 43]. Gluconeogenesis from pyruvate and glutamine is also inhibited by the addition of adenosine [42]. Similarly, adenosine inhibits gluconeogenesis from lactate in guinea pig and chicken hepatocytes [44]. Alterations in glucose levels could account for the decrease in UDPGA levels seen with adenosine; however, this does not appear to be the sole mechanism responsible for the decreases in UDPGA content. Addition of graded concentrations of glucose to hepatocyte suspensions prior to incubation with adenosine did not reverse completely the effects of adenosine on glucuronidation. Measurement of the content of glucose in the cells also showed that adenosine only slightly (9%) decreased glucose levels in the cells (Table 2). DBcAMP, at concentrations of 500 and 100 μM, produced small but statistically significant decreases in glucose levels. This finding was unexpected in that cAMP has been shown to stimulate gluconeogenesis [10].

Both adenosine and the two higher concentrations of DBcAMP decreased glycogen content. This would be expected in that increases in cAMP stimulate glycogenolysis in the liver [10]. Marchand *et al.* [45] found that adenosine inhibits glycogenolysis by 50% in isolated rat hepatocytes. This observation is surprising in that cAMP levels were increased by 50% in their study which would suggest enhanced not decreased glycogenolysis. However, this discrepancy between studies may reflect differences in initial glycogen levels. The hepatocytes used in the present study were prepared from fasted rats, whereas in the study by Marchand *et al.* [45] the hepatocytes were from fed rats. Glycogen levels are decreased markedly in fasted animals as compared to fed rats [46, 47]. Cells from fasted rats have also been shown to have a higher sensitivity to glucose for activation of glycogen synthetase *a* and inactivation of phosphorylase *a* (the enzyme that inhibits glycogen synthesis) than cells from fed rats [48]. In the livers of food-deprived rats, there is also a higher initial content of synthetase [48, 49]. Hue *et al.* [48] suggests that, in the livers from fasted rats, synthesis of glycogen could operate at least at a low rate, simultaneously with degradation. Therefore, the apparent differential effects of adenosine on glycogenolysis in fed and fasted rats could be caused by alterations in glycogen synthesis which is occurring at 5.5 mM glucose concentrations in fasted animals but not in fed rats [48].

Another possible explanation for the differences in glycogen levels may be related to the sex of the animals utilized. Marchand *et al.* [45] used female rats, whereas male rats were used in the present study. Studer and Borle [50] have demonstrated differences between male and female rats in the regulation of hepatic glycogenolysis. The age [51] and the species [52, 53] of the animal may also play a role in the control of glycogenolysis.

Isolated perfused rat liver [54] and *in vivo* [55] studies have also shown differences in hepatic glycogen content in response to adenosine. In the isolated perfused liver the rate of glucose formation from lactate was not influenced by a concomitant synthesis or breakdown of glycogen. An insignificant increase (4%) in glycogen content has been observed with no overall change in gluconeogenesis with adenosine preperfusion [54]. In contrast, adenosine pretreatment in rats brings about a 2-fold increase in liver glycogen content *in vivo* [55]. These studies examined the effects of adenosine alone on glycogen content, whereas in the present study both adenosine and pNP were included in the incubation. Therefore, it is possible that the presence of pNP altered the effect of adenosine on glycogen content due to the relationship between carbohydrate reserve and glucuronidation.

If the decreases in glycogen content are due to stimulation of glycogenolysis by cAMP, then free glucose levels in the cells would be expected to increase; however, they do not. Marchand *et al.* [45] also found glucose consumption to be decreased when hepatocytes were incubated with adenosine. The decreases in free glucose content after 30-min incubations with adenosine or DBcAMP suggest that glucose is being shunted to another pathway—possibly the glucuronic acid pathway—due to the decreases in UDPGA seen with these compounds as well as utilization of available UDPGA to conjugate the pNP present during these incubations.

Determinations of the lactate and pyruvate levels in cells incubated with DBcAMP further supports

rapid utilization of free glucose in the cells. DBcAMP, at concentrations of 100 and 50 μM, decreased cellular levels of both lactate and pyruvate, suggesting that gluconeogenesis is occurring due to increases in cAMP. The magnitude of the changes in both levels was similar, and thus the [lactate]/[pyruvate] ratio as well as the NAD^+/NADH ratio was not changed significantly from the control value. These findings are supported by an earlier study [56] in which DBcAMP (50 μM) significantly decreased both lactate and pyruvate levels in isolated rat hepatocytes without altering the NAD^+/NADH ratio. At the highest concentration of DBcAMP, the [lactate]/[pyruvate] ratio was only slightly increased and the NAD^+/NADH ratio slightly decreased. The absence of change in the levels of lactate and pyruvate at the highest concentration of DBcAMP cannot be explained at this point. Adenosine caused a much greater increase in the [lactate]/[pyruvate] ratio and thus a greater magnitude of change in the NAD^+/NADH ratio. Similar decreases in NAD^+/NADH ratios in response to adenosine have been demonstrated in isolated hepatocytes [42, 45]; however, the changes in the present study (62%) were of a greater magnitude than seen by Lund *et al.* (38%) [42]. These differences in magnitude may be explained by the addition of lactate (10 mM) and pyruvate (1 mM) to the hepatocytes suspension used by Lund *et al.* [42].

Alterations in the redox state of the cells explains the decrease in UDPGA levels seen with adenosine. UDP-glucose dehydrogenase is an NAD^+-requiring enzyme; therefore, the decrease in NAD^+ leads to decreased activity of UDP-glucose dehydrogenase and reduced synthesis of UDPGA. Other substances, such as ethanol, lactate, and sorbitol, that decrease the NAD^+/NADH ratio have also been shown to decrease UDPGA synthesis and, therefore glucuronide formation [57]. Thus, the major mechanism responsible for the effects of adenosine on glucuronidation appears to be a disruption of the redox state.

While DBcAMP decreased the cellular content of UDPGA and thus inhibited glucuronidation, it did not decrease the NAD^+/NADH ratio as seen with adenosine. This discrepancy could suggest a different mechanism of action. However, since adenosine has been shown to increase cAMP levels [19] in isolated hepatocytes, it is also possible that cAMP may directly inhibit UDP-glucose dehydrogenase. Although no inhibition of UDP-glucose dehydrogenase by adenine nucleotides has been reported, adenine nucleotides have been shown to inhibit the sulfotransferase enzyme [58]. The nonspecific inhibition of sulfotransferase activity appeared to involve competition with PAPS, the cofactor required for sulfation. Thus, it is possible that an increase in cAMP could disrupt reactions that are dependent on structurally similar analogs, such as the synthesis of UDPGA which requires NAD^+ for the dehydrogenation of UDP-glucose.

Both adenosine and the two highest concentrations of DBcAMP (500 and 100 μM) significantly increased the intracellular concentrations of total adenine nucleotides (Table 4). The increase in total nucleotides with adenosine is consistent with that found by Lund *et al.* [42] and Marchand *et al.* [45]. Only adenosine caused a significant increase in the [ATP]/[ADP] ratio; however, the increase was only slightly higher than the normal values of 2 to 3. Furthermore, Thurman and Kauffman [59] have suggested that cellular energetics do not normally limit glucuronidation. Lund *et al.* [42] also found the [ATP]/[ADP] ratio increased, but no clear relationship could be established between the altered ratio and the inhibition of gluconeogenesis. Similarly, Marchand *et al.* [45] was unable to correlate the increase in [ATP]/[ADP] with the decrease in the NAD^+/NADH ratio. No satisfactory answer can yet be offered to explain the inhibition of energy-requiring processes in the liver when ATP is in excess. Thus, the increases in adenine nucleotide content do not appear to be responsible for the actions of adenosine on glucuronidation.

Although no *in vivo* Phase II studies have been performed, Ross *et al.* [60] have examined the effects of adenosine on Phase I metabolism. Adenosine pretreatment was found to decrease aminopyrine demethylation in both male and female rats. Similarly, equimolar DBcAMP pretreatment brought about even greater inhibition in the metabolism of aminopyrine, while also decreasing aniline biotransformation. In addition, reductions were seen when DBcAMP was added directly to liver slices and perfused livers [61–63]. Thurman and Kaufman [59] have suggested that mixed-function oxidation and glucuronidation are in some manner coordinately regulated; therefore, it would be of interest to examine the effects of adenosine *in vivo* to determine if the pattern of *in vitro* and *in vivo* inhibition of Phase I metabolism by DBcAMP also occurs with Phase II metabolism.

In summary, adenosine apparently decreased the synthesis of UDPGA in the hepatocytes by decreasing the NAD^+/NADH ratio, thus inhibiting UDP-glucose dehydrogenase. Moreover, carbohydrate reserve appeared to be involved because additions of graded concentrations of glucose lessened the inhibition of glucuronidation mediated by adenosine. DBcAMP also decreased UDPGA levels in isolated hepatocytes without altering the NAD^+/NADH ratio. This finding may suggest a different mechanism of action for DBcAMP. Since adenosine has been shown to increase cAMP levels through its interaction with extracellular receptors in hepatocytes, studies are ongoing to explore the possibility that the inhibition of glucuronidation caused by adenosine was receptor-mediated.

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