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SHORT COMMUNICATIONS

High amounts of glucose and insulin inhibit *p*-nitrophenol conjugation in mouse hepatocytes

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Conjugation with UDP-glucuronic acid (UDPGA), a major pathway in the second phase of biotransformation of foreign and endogenous compounds, is catalysed by various UDP-glucuronosyltransferase (GT) isozymes. The UDPGA supply plays a crucial role in the regulation of glucuronidation [1]. A linear relationship has been observed between UDPGA levels and the glucuronidation of planar phenols in isolated hepatocytes [2]. UDPGA supply for glucuronidation in the liver depends on (i) the NAD/NADH redox state, (ii) the energy state of the cell, (iii) activity of enzymes participating in UDPGA synthesis and (iv) the carbohydrate reserves [3, 4]. Furthermore, in accordance with these observations, it has been reported that UDPGA for glucuronidation in the liver is provided at the expense of glycogenolysis [5]. Thus, drug glucuronidation is a glycogen consuming process in the liver. Inhibition of glycogenolysis and/or stimulation of glycogen synthesis by fructose [6] has inhibited the conjugation of *p*-nitrophenol in isolated hepatocytes [5]. The question arises, do important and physiological stimulators of glycogen synthesis, such as insulin or glucose, affect conjugation of *p*-nitrophenol? Moreover, it is not clear whether UDPGA supply is a rate determining factor for glucuronidation in hepatocytes from fed animals under normal conditions. In starvation the depleted glycogen stores have been shown to limit the cofactor supply for glucuronidation [5, 7]. The purpose of the present study was to investigate the role of cofactor supply in the regulation of glucuronidation in the fed state.

Materials and Methods

Male CFLP mice (30–35 g body wt) were used throughout the experiments. Fasted mice were deprived of food for 24 hr before use. Isolated hepatocytes were prepared as described earlier [8]. Viability of the cells controlled by the Trypan blue exclusion test was about 90%. Hepatocytes (5×10^6 cells/mL) were incubated in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 1% albumin, 8.5 mM glucose (if otherwise is not indicated), 5 mM pyruvate and amino acids necessary for protein synthesis (1 mM of each) under constant bubbling of gas (O_2:CO_2, 95:5, v/v) at 37°.

Conjugation of *p*-nitrophenol in hepatocytes was investigated by measurement of disappearance of *p*-nitrophenol as described previously [9]. The formation of glucuronide and sulfate conjugates of *p*-nitrophenol was determined enzymatically.

Isolated hepatocytes were permeabilized with 0.005% saponin. After permeabilization more than 95% of the cells became permeable for Trypan blue. The integrity of endoplasmic reticulum membranes in permeabilized cells was checked by measuring the latency of mannose-6-phosphatase activity [10].

Activity of glycogen phosphorylase *a* was determined by Stalmans and Hers [11]. cAMP content was measured by radioimmunoassay (RIA). Microsomes were prepared by differential centrifugation. Activity of *p*-nitrophenol GT was measured according to Bock *et al.* [12]. Protein content was measured by the method of Lowry *et al.* [13]. Significance was determined using a Student's *t*-test (two-tailed) for paired samples.

Chemicals: collagenase type IV, β-glucuronidase type IX and arylsulfatase type VIII were purchased from Sigma, insulin from Novo, cAMP RIA kit from Chemapol.

Results and Discussion

Isolated hepatocytes were prepared from fed mice and the disappearance of *p*-nitrophenol was determined. At 100 μM substrate concentration *p*-nitrophenol is conjugated mainly (70%) with UDPGA (Fig. 1, inset), in accordance with previous observations on isolated rat and mouse hepatocytes [5, 14, 15]. The conjugation of *p*-nitrophenol

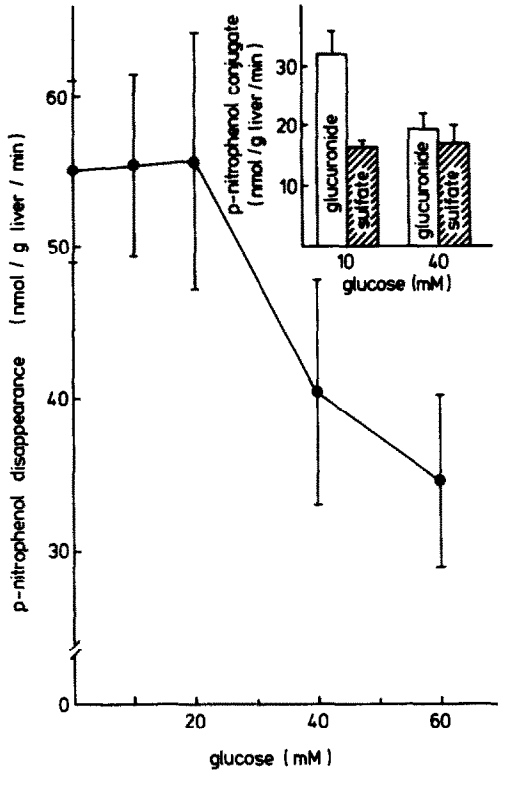

Fig. 1. Effect of glucose on *p*-nitrophenol conjugation in isolated hepatocytes. Isolated hepatocytes were incubated in the presence of various concentrations of glucose for 30 min and the conjugation of *p*-nitrophenol was determined. Inset: Effect of 40 mM glucose on *p*-nitrophenol glucuronide and sulfate formation. Mean (N = 6). Vertical bars indicate ± SD.

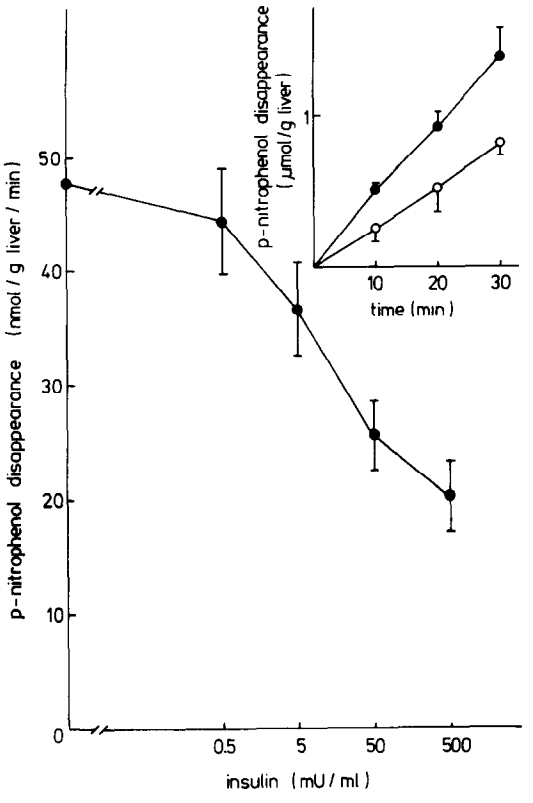

Fig. 2. Effect of insulin on *p*-nitrophenol conjugation in isolated hepatocytes. Isolated hepatocytes were incubated in the presence of various amounts of insulin for 10 min and conjugation of *p*-nitrophenol was determined. Inset: in a series of experiments the cells were incubated in the presence of 50 mUnits/mL of insulin (○) or in the absence of it (●) and the conjugation of *p*-nitrophenol was determined. Mean (N = 4). Vertical bars indicate ± SD.

was linear in time (Fig. 2, inset). Addition of glucose above 20 mM was inhibitory on *p*-nitrophenol conjugation (Fig. 1). This inhibition was due to the repression of glucuronidation, while the formation of *p*-nitrophenol sulfate was unaltered (Fig. 1, inset). It is noted that the absence of glucose did not influence the rate of *p*-nitrophenol conjugation. Insulin also caused inhibition of conjugation of *p*-nitrophenol. The inhibitory effect of insulin was dose dependent (Fig. 2).

The inhibitory effect of insulin and glucose on *p*-nitrophenol conjugation confirms the view that the primary source of UDPGA is the glycogenolysis. Both insulin and high amounts of glucose have been shown to inhibit glycogen breakdown (and to stimulate glycogen synthesis) [16, 17]. On the other hand, the stimulation of glycogen synthesis by glucose causes a marked decrease in the concentration of UDP-glucose in the liver [18]. It is tempting to suppose that there is a competition for UDP-glucose between glycogen synthesis and glucuronidation. Our previous observations concerning the inhibitory action of fructose on *p*-nitrophenol conjugation are in accordance with this assumption [5].

It is well known that the rate of *p*-nitrophenol conjugation is maximal at about 100 μM substrate concentration in isolated hepatocytes and also in perfused liver. However, at higher concentrations a significant decrease of conjugation has been observed [14]. This effect was caused mainly by the repression of glucuronidation [14], but the explanation of this phenomenon was unknown. In our experiments the maximal rate of *p*-nitrophenol conjugation was attained in the presence of 100 μM *p*-nitrophenol in accordance with previous observations [4, 14]. The conjugation of *p*-nitrophenol had already decreased in the presence of 200 μM *p*-nitrophenol (Table 1). A possible explanation of this observation would be that *p*-nitrophenol at this concentration might decrease the activity of *p*-nitrophenol GT. However, a normal saturation curve of *p*-nitrophenol GT was obtained for up to 1 mM *p*-nitrophenol measured in murine hepatic microsomes and the K_m value for GT_1 was 0.230 mM (data not shown). These findings do not support the above explanation. As the primary source of UDPGA is glycogenolysis the effect of *p*-nitrophenol on the activity of glycogen phosphorylase *a* was also determined. *p*-Nitrophenol (200 μM) had already caused a decrease in phosphorylase *a* activity after 5 min (Table 1). This decrease of phosphorylase *a* activity was shown for up to 30 min incubation in the presence of 200 μM *p*-nitrophenol. It is also noted that *p*-nitrophenol even at 5 mM concentration did not influence the determination of phosphorylase *a* (and also *a* + *b*) activity (data not shown).

The decreasing effect of *p*-nitrophenol on its own conjugation and the simultaneous decrease of phosphorylase *a* activity (Table 1) supports our previous suggestion that glycogen is the source of UDPGA for glucuronidation.

Glycogenolysis in the liver is under a positive cAMP (and Ca^{2+}) dependent control. *p*-Nitrophenol decreased the cAMP content of cells both under normal conditions and also after stimulation by glucagon in a 5 min incubation (Table 1). *p*-Nitrophenol has been also reported as

Table 1. Effect of *p*-nitrophenol on activity of glycogen phosphorylase *a*, on cAMP level and on its own conjugation in isolated hepatocytes

p-Nitrophenol (μM)	*p*-Nitrophenol disappearance (nmol/g/min)	cAMP content (pmol/g liver)	cAMP content in the presence of 10^{-7} M glucagon (pmol/g liver)	Glycogen phosphorylase *a* activity (nmol phosphate/min/mg protein)
0	—	360 ± 4	1443 ± 52*	54.0 ± 2.9
100	38.0 ± 3.5	317 ± 16	1137 ± 46	48.5 ± 4.5
200	18.0 ± 5.4*	291 ± 38	836 ± 28*	28.4 ± 6.2*
500	11.8 ± 3.2*	218 ± 44*	300 ± 25*	29.6 ± 7.8*

Isolated hepatocytes were incubated in the presence of various amounts of *p*-nitrophenol. Disappearance of *p*-nitrophenol was determined after 10 min and activity of phosphorylase *a* and amount of cAMP after 5 min incubation. Values are means ± SD (N = 4–9).

* Different from 100 μM *p*-nitrophenol at $P < 0.01$ level.

Table 2. Effect of UDP-glucuronic acid on *p*-nitrophenol conjugation in saponin-permeabilized hepatocytes

	p-Nitrophenol disappearance (nmol/g liver/min)	
	Fed	Fasted
Untreated	45.8 ± 6.3 (12)	15.6 ± 3.6† (6)
Saponin-treated	16.7 ± 6.6* (8)	6.8 ± 3.1*† (6)
Saponin-treated + UDP-glucuronic acid (3 mM)	66.7 ± 6.7* (10)	61.5 ± 10.9* (6)

Saponin-treated and intact hepatocytes from fed or fasted mice were incubated in the presence and absence of 3 mM UDP-glucuronic acid for 30 min. *p*-Nitrophenol conjugation was measured.

Values are means ± SD (N).

* Different from the untreated at $P < 0.01$ level.

† Different from fed at $P < 0.01$ level.

inhibiting intracellular Ca^{2+} mobilization in platelets [19]. It should be emphasized that *p*-nitrophenol in the applied concentrations did not cause a significant loss in the viability of hepatocytes up to 30 min incubation. Since in our experiments the rate of glycogenolysis could affect the rate of glucuronidation, we supposed that UDPGA supply may be rate limiting in the conjugation even in the fed state. To confirm this hypothesis we investigated the effect of exogenously added UDPGA on the conjugation of *p*-nitrophenol in permeabilized hepatocytes. There are several, though conflicting, reports on the effect of starvation on glucuronidation also depending on the types and doses of drugs examined [20–23]. Starvation caused a significant reduction of the *p*-nitrophenol conjugating capacity of the cells (Table 2). After permeabilization a very low rate of conjugation could be measured, even in hepatocytes isolated from fed animals, presumably due to the leakage of low molecular mass compounds from the cells. It should be emphasized that the integrity of the endoplasmic membrane was mostly preserved, the mannose-6-phosphatase activity of the saponin-treated cells was lower than 10% with respect to the activity of fully disrupted cells in each preparation. Addition of 3 mM UDPGA caused a 4-fold increase in conjugation in cells originating from 24 hr starved mice, and elevated the rate of conjugation over the value measured in hepatocytes prepared from fed mice. In the fed state in intact cells about two-thirds of the conjugates are glucuronide and one-third is sulfate (Fig. 1, inset). In permeabilized cells, where almost exclusively exogenous UDPGA is available, the rate of conjugation is higher than in intact cells (Table 2). These findings suggest that in accordance with previous assumptions in intact cells glucuronidation works below its maximal capacity.

Based on our findings it is concluded that (i) cofactor supply for glucuronidation is related to the actual state of glycogen metabolism; (ii) the regulation of phosphorylase *a* activity is closely correlated with the rate of glucuronidation; and (iii) UDPGA level is rate limiting for glucuronidation of high rate not only in starvation but also in the fed state.

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Effects of morphine tolerance-dependence and abstinence on κ-opiate receptors of rat brain and spinal cord

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Several attempts have been made to understand the possible role of opiate receptors in morphine tolerance-dependence and abstinence processes; however, the picture is far from clear. As indicated in a previous publication [1], factors which could give rise to different results include the degree of tolerance-dependence induced, the animal species used, the selectivity of various ligands used to characterize the receptors, whether or not the animals were undergoing abstinence and whether whole brain or various brain regions were used for the study of a specific receptor. In more recent studies using D-Ala^2,MePhe^4,Gly-ol^5-enkephalin (DAMGO), a highly selective ligand for the μ-opiate receptor [2], it has been shown that μ-receptors are down-regulated in the spinal cord and specific areas of the brain of morphine tolerant-dependent rats and guinea pigs [1, 3]; however, they are unaffected in the morphine abstinence process.

Little is known about the role of κ-opiate receptors in morphine tolerance-dependence and abstinence processes. Several lines of evidence suggest that endogenous or exogenous κ-opiate receptor agonists antagonize the actions of morphine in non-tolerant animals but potentiate them in morphine tolerant-dependent animals [4–7]. The intensity of dynorphin-(1-13)-induced catalepsy was found to be greater in sufentanil (a μ-opiate agonist) -tolerant rats than in non-tolerant rats [8]. Therefore, it can be expected that there may be changes in κ-opiate receptors induced by chronic treatment with morphine.

The present studies were undertaken to determine the binding of [^{3}H]ethylketocyclazocine ([^{3}H]EKC) and [^{3}H]-U-69,593 to membranes of discrete brain regions and spinal cord of morphine tolerant-dependent and abstinent rats. In addition, the effect of U-50,488H, a κ-opiate receptor agonist, on the thermoregulatory behavior in non-tolerant and morphine-tolerant rats also was determined.

Materials and Methods

Animals. Male Sprague–Dawley rats weighing 225–250 g (Sasco-King Animal Co., Oregon, WI) were acclimatized to a room with controlled ambient temperature (23 ± 10%), and a 12-hr dark–light cycle (light 6:00 a.m. to 6:00 p.m.). The animals were housed under these conditions for at least 4 days prior to being used and were given food and water continuously.

Chemicals. Morphine and placebo pellets, U-50,488H, U-69,593 and [^{3}H]EKC (sp. act. 35.0 Ci/mmol) were supplied by the National Institute on Drug Abuse, Rockville, MD, through the courtesy of Mr. Robert Walsh. [^{3}H]U-69,593 (sp. act. 58 Ci/mmol) was purchased from the New England Nuclear–Du Pont Corp., Boston, MA. Unlabeled levorphanol was a gift from the Hoffmann-LaRoche, Nutley, NJ.

Induction of tolerance to and physical dependence on morphine. Rats were rendered tolerant to, and physically dependent on, morphine by s.c. implantation of six morphine pellets during a 7-day period as described previously [9–11]. On the evening of day 7, the pellets were removed from one group of rats and they were killed 18 hr later (abstinent rats). The other set of rats in which pellets were left intact were killed on day 8 (tolerant-dependent but non-abstinent rats). The spinal cord (cervical to lumbar region) and brain regions (amygdala, hippocampus, hypothalamus, corpus striatum, midbrain, pons and medulla, and cortex) were isolated.

Determination of the binding of [^{3}H]*EKC and* [^{3}H]*U-69,593 to opiate receptors in discrete brain regions and*