An estimate of the effect of acute exsanguination on catecholamine and glucoregulatory hormone concentrations in the newborn rat

R. D. Jansen and H. C. Moorehead

Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana, USA

Summary. We have determined the effect of acute exsanguination on plasma concentrations of glucose, insulin, glucagon, dopamine, epinephrine and norepinephrine in neonatal Sprague-Dawley rats. This was done by comparing concentrations of these substances in aliquots of blood obtained within 10 s of the initial bleeding to those in blood obtained over the next 50 s of the blood drawing process. Concentrations of glucose and insulin showed no change between early and late samples. Glucagon concentrations showed variable responses dependent upon the age of the animal. Concentrations were unchanged at birth, but increased 22% and 58% at 1 and 6 h of

age respectively. Catecholamine concentrations increased greatly (57 to 215%) between blood aliquots regardless of age at the time of sampling. These findings indicate that hormonal responses occur during the relatively brief period of blood drawing in Sprague-Dawley rats, pointing out further limitations inherent in the use of small animals for acute metabolic and hormonal studies.

Key words: Glucoregulatory hormones, newborns, haemorrhage.

Fetal and neonatal rat pups have been subjects of numerous studies characterizing glucoregulatory adaptations in the perinatal period. Girard et al. [1] and Girard and Sperling [2] have reviewed the results of many such studies. Due to the small size of rats at this stage of development, such studies uniformly involve the collection of only one sample of blood from individual animals at a single point in time. This is due to the fact that the measurement of circulating metabolic fuels or hormones in this animal requires the collection of a substantial quantity of blood relative to the pup's size. Often essentially complete exsanguination is necessary if concentrations of hormones or of multiple substrates are to be assayed. However, the effects of the blood drawing process itself on circulating concentrations of these substances has not been evaluated. In larger animals haemorrhage is recognized to acutely affect concentrations of some of metabolites and hormones [3, 4]. Such metabolites and hormones often have been of interest to those using newborn rats as subjects. This report provides an estimate of the effect of the blood drawing process on plasma concentrations of glucose, insulin, glucagon, dopamine, epinephrine and norepinephrine in neonatal rats.

Materials and methods

Primiparous timed-pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN, USA) were allowed unrestricted access to water and a standard rat chow (Purina, St. Louis, MO, USA). The duration of

pregnancy was estimated from the assumed time of ovulation, which usually occurred between midnight and 03.00 h the night of cohabitation with a male. At approximately 21.5 days gestation (term = 22), maternal rats were delivered by hysterotomy after they were killed by cervical dislocation. Pups were carefully dried and their placentas removed. A heating blanket and a heat lamp were used at delivery and at the subsequent times of blood drawing to avoid cold stress. Following delivery, the pups were transferred to a humidicrib maintained at 36 °C, 70% relative humidity. The pups had carcass weights between 5.2 and 6.0 g (mean $\pm$ standard deviation, 5.53 ± 35 g). These weights were in close agreement with those of pups born spontaneously at term in our laboratory. Litters were randomly utilized for insulin, glucagon or catecholamine determinations. Within each litter, littermates were selected so as to have roughly equivalent numbers of pups [3–4] at each sampling time.

Blood was collected from individual pups within 2 min of birth, at 1 h, or a 6 h age. Pups were gently removed from the humidicrib and an axillary incision was made to transect the axillary vessels. Free-flowing blood was collected in two parts: **(A)**, blood collected in the first 10 s after incision (early collection); and **(B)**, blood collected over the subsequent 50 s (late collection). This division provided essentially equal aliquots of blood. There was no difference in bleeding rates among the three times of sampling. Following the blood drawing the pups were killed by decapitation.

All blood samples were collected in chilled heparinized capillary tubes. Blood for insulin and glucagon assays was collected with Trasyol, 25:1 v/v, and immediately centrifuged. Plasma was rapidly frozen and stored at -70 °C until the time of assay. Blood for plasma catecholamine determinations was collected with EGTA [ethylene glycol-bis (β -amino-ethyl ether) N,N,N',N'-tetra-acetic acid], centrifuged immediately, and the plasma frozen and stored at -70 °C.

Plasma glucose concentrations were determined by a glucose oxidase technique (Beckman Glucose Analyzer, Beckman Instruments, Fullerton, CA, USA). Plasma catecholamine concentrations were determined using a radioenzymatic technique (Cat-a-Kit, Upjohn Diag-

nostics, Kalamazoo, MI, USA) [5]. Plasma insulin was measured using a double antibody radioimmunoassay system employing rat insulin standards (Novo Laboratories, Inc., Copenhagen, Denmark) [6]. Plasma glucagon concentrations were determined with a double antibody radioimmunoassay using porcine glucagon as standard (Eli Lilly Company, Indianapolis, IN, USA) and employing 30K antibody (obtained from Dr. R. Unger) [7]. The assay system was modified for small sample volumes as by Bussey et al. [8].

Paired A and B samples (early and late collections respectively) were assayed together for each measured substance. All insulin concentrations were determined in a single assay, as were glucagon concentrations.

Because the plasma volumes obtained were small, only one hormonal assay (insulin, glucagon, or catecholamines) was performed on each sample. Where volumes allowed, plasma glucose was determined in each specimen. Also because of sample volume limitations, glucagon determinations were done using plasma samples from paired littermates. That is, equal aliquots of early- and late-drawn plasma were paired with those of a littermate and the plasma specimens thawed, combined, mixed well, and then assayed.

Coefficients of intra- and inter-assay variation in our laboratory are 3% and 11% for glucose and catecholamines respectively. Intra-assay coefficients of variation are 10% and 13% for insulin and glucagon respectively.

Statistical analysis

Paired data were analyzed by two-tailed paired Student's *t*-tests, and compared early collection blood (A) with late collection blood (B) at each time of drawing. Data are presented as means $\pm$ one standard deviation of the mean (mean $\pm$ SD). *P* values of greater than 0.05 were considered as insignificant (NS).

Results

A summary of the results is given in Table 1. Plasma glucose concentrations did not show statistically significant differences between the early (A) and late (B) paired collection samples at any of the three sampling times. Early and late collection plasma insulin concentrations similarly were not different at any of the sampling times.

However, statistically significant increases in concentrations of glucagon and catecholamines were noted when early and late samples were compared. Plasma glucagon showed mean increases of 22% and 58% between early and late specimen pairs at 1 h and 6 h respectively ($p < 0.05$), although at birth glucagon concentrations in A and B samples were not different. Plasma epinephrine concentrations were greatly increased in B samples, with increases of 122%, 215%, and 165% above the corresponding A samples at birth ($p < 0.05$), 1 h ($p < 0.05$) and 6 h ($p < 0.01$) respectively. Norepinephrine concentrations also increased between sample pairs, although to a lesser extent than epinephrine. Mean increases in norepinephrine were 69% at birth ($p < 0.05$), 58% at 1 h ($p < 0.01$), and 106% at 6 h ($p < 0.01$). Plasma dopamine concentrations similarly increased between sample pairs. Mean increases were 57% at birth ($p < 0.05$), 132% at 1 h ($p < 0.01$), and 96% at 6 h ($p < 0.01$).

Table 1. Plasma glucose, hormone and catecholamine concentrations in early (A) and late (B) blood collection samples

	Time of blood drawing					
	Birth		1 h		6 h	
Glucose (mol/l)						
A (mean $\pm$ SD)	3.36 $\pm$	0.73	1.01 $\pm$	0.41	4.21 $\pm$	0.75
B (mean $\pm$ SD)	3.41 $\pm$	0.73	0.99 $\pm$	0.37	4.29 $\pm$	0.74
^a Mean difference (% $\pm$ %SD)	1.5 $\pm$	5.0	-2.0 $\pm$	24.1	2.1 $\pm$	4.3
^b <i>n</i> =	23		22		20	
<i>p</i>	NS		NS		NS	
Insulin (μU/ml)						
A (mean $\pm$ SD)	85.6 $\pm$	22.6	19.6 $\pm$	8.3	8.9 $\pm$	4.6
B (mean $\pm$ SD)	83.6 $\pm$	22.0	20.3 $\pm$	9.5	8.7 $\pm$	2.1
Mean difference (% $\pm$ %SD)	-0.6 $\pm$	19.0	0.25 $\pm$	1.1	18.5 $\pm$	52.5
<i>n</i> =	12		13		14	
<i>p</i>	NS		NS		NS	
Glucagon (pg/ml)						
A (mean $\pm$ SD)	190.4 $\pm$	55.6	182.0 $\pm$	49.2	139.4 $\pm$	72.3
B (mean $\pm$ SD)	185.7 $\pm$	50.3	217.8 $\pm$	58.6	183.7 $\pm$	27.6
Mean difference (% $\pm$ %SD)	-0.7 $\pm$	14	22 $\pm$	27	58 $\pm$	64
<i>n</i> =	12		11		11	
<i>p</i>	NS		<.05		<.05	
Dopamine (pg/ml)						
A (mean $\pm$ SD)	753 $\pm$	135	312 $\pm$	64	391 $\pm$	52
B (mean $\pm$ SD)	1185 $\pm$	136	725 $\pm$	82	770 $\pm$	175
Mean difference (% $\pm$ %SD)	57 $\pm$	20	132 $\pm$	24	96 $\pm$	47
<i>n</i> =	10		9		10	
<i>p</i>	<.01		<.001		<.01	
Epinephrine (pg/ml)						
A (mean $\pm$ SD)	5575 $\pm$	4416	801 $\pm$	291	1489 $\pm$	800
B (mean $\pm$ SD)	8466 $\pm$	3842	2251 $\pm$	897	3545 $\pm$	1654
Mean difference (% $\pm$ %SD)	122 $\pm$	151	215 $\pm$	164	164 $\pm$	90
<i>n</i> =	10		9		10	
<i>p</i>	.05		<.05		<.001	
Norepinephrine (pg/ml)						
A (mean $\pm$ SD)	2876 $\pm$	1331	901 $\pm$	340	801 $\pm$	473
B (mean $\pm$ SD)	4489 $\pm$	1553	1294 $\pm$	248	1402 $\pm$	527
Mean difference (% $\pm$ %SD)	69 $\pm$	46	58 $\pm$	53	107 $\pm$	96
<i>n</i> =	10		9		10	
<i>p</i>	<.05		<.01		<0.1	

^a Mean difference (%) calculated as $\frac{B-A}{A} \times 100$; ^b *n* = number of sample pairs assayed

Discussion

The newborn rat has been the subject of extensive study with regard to glucoregulatory events occurring in the immediate neonatal period. Key to the achievement of glucose homeostasis is a fall in insulin from high fetal levels to low basal concentrations, as well as a surge in glucagon secretion [1, 9, 10]. These hormonal events occur within the first hour of life, and the surge in glucagon has been related to the increase in catecholamines occurring at delivery [2, 11, 12]. The above studies have used non-anaesthetized animals whose blood was collected either after incision of axillary vessels or from the neck at decapitation. Several investigations have documented that the interpretation of data obtained from such "acute preparations" used for physiologic or meta-

bolic studies potentially may be fraught with error [13–15].

The study reported here was designed to determine whether glucose, insulin, glucagon or catecholamine concentrations are affected by the blood drawing process. This was done by comparing concentrations of these substances in blood obtained in the first 10 s of the blood drawing process with those in blood obtained over a subsequent 50-s period. This division of the collection time was selected because it resulted in approximately equal quantities of blood being obtained in each portion of the paired samples. Also, the free flow of blood is essentially complete by the end of 1 min. A technique using axillary incision was used, since the blood obtained would be relatively free from contamination. Blood collected from the neck after decapitation is necessarily admixed with cerebrospinal fluid and pharyngeal secretions. Finally, three sampling times were evaluated in order to determine whether any observable effects of the blood drawing might be influenced by the stage of adjustment to extrauterine life. At birth, insulin [9, 10] and catecholamines [11] are high, subsequently falling rapidly. At 1 h of age, hypoglycaemia is evident, glucagon has surged and insulin is at basal levels [1, 9, 10]. At 6 h, glucose concentrations have stabilized, as have concentrations of insulin, glucagon and catecholamines [1, 9–12].

The data presented indicate that plasma glucose concentrations are equivalent in early and late samples regardless of the timing of the blood draw. Similarly, no detectable change in insulin concentration occurred during the blood sampling, regardless of whether concentrations were high as at birth or low as in the later times of sampling. Although "stress" situations are well known to lead to increased glucose and decreased release of insulin [16, 17], the duration of the total sampling time in this study was relatively brief and apparently insufficient for such variations to be detected.

Increases in glucagon and catecholamines were detected. Glucagon concentrations have been recognized to increase in response to exsanguinating haemorrhage accomplished over 40–70 min [3, 4]. Adrenergic stimulation of glucagon secretion has been considered to be the mechanism responsible for the increase in glucagon concentration [4]. Our data indicate that glucagon concentrations increase within the relatively brief period of the blood collection technique used here, during which time exsanguination rapidly occurs. Since catecholamine concentrations also increased greatly during the blood drawing process, it is likely that adrenergic effects were responsible for the increase in glucagon observed at the 1- and 6-h sampling times. The apparent failure of glucagon to increase with exsanguination at birth may relate to the finding that catecholamine concentrations were already extremely high. Thus, the further increase in catecholamines with haemorrhage may not have provided an additional adrenergic stimulation for glucagon secretion.

Glucagon concentrations at 1 h did not reflect the postnatal surge of this hormone as has been well described [1, 9–12]. In an earlier study in our laboratory [19], glucagon concentrations in non-asphyxiated rats did surge during the first hour of life, being substantially elevated at 20 and 40 min, but returned by 60 min to a level similar to that at birth. In the present study, if values from early and late specimens are averaged, the mean glucagon concentration at 1 h of 206 ± 41 pg/ml is not statistically different from the mean concentration at birth of 190 ± 50 pg/ml ($p = 40$ by 2-tailed non-paired Student's *t*-test). These values are in close agreement with our earlier results [19].

Catecholamine concentrations also increased in response to blood drawing. Epinephrine and norepinephrine concentrations were consistently higher in blood obtained in the later portion of each sampling. The fetus and neonate of various species are capable of responding to stress with rapid increases in catecholamines [11, 18–21]. Our findings indicate that the neonatal rat responds to the stress of blood drawing with a very large increase in circulating catecholamines. The increase in epinephrine was proportionately greater than the increase in norepinephrine.

Our findings indicate further limitations inherent in the use of small animals for acute metabolic or hormonal studies. Although it is reassuring that glucose and insulin concentrations apparently did not change over the course of the blood drawing process, the glucagon and catecholamine data indicate that concentrations of these latter substances do undergo change during exsanguination. Also, as suggested by the variable effects on glucagon, the extent to which these changes occur may vary with the time at which samples are obtained in relation to birth. Finally, it should be recognized that the early-collected portion of the specimen pairs cannot be taken to be an absolute basal value, as even the brief handling necessary to obtain blood may potentially produce alterations in the concentrations of substances to be assayed.

Acknowledgement. Supported in part by 2P60AM20542 from the National Institute of Health and the Riley Memorial Association.

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Received: 16 November 1985
and in revised form: 2 April 1986

Dr. Robert D. Jansen
Department of Pediatrics
Prentice Women's Hospital
Room 464
333 East Superior Street
Chicago IL 60611
USA