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Determination of heat-exposure effects on the concentration of catecholamines in bovine plasma and milk

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method with electrochemical detection has been adapted for the determination of picogram concentrations of norepinephrine and epinephrine in bovine plasma and milk. This method has been used to monitor the levels of these catecholamines when lactating cows are exposed to heat stress under controlled conditions. In response to heat stress, epinephrine concentrations in milk and plasma were similar. However, norepinephrine concentrations in milk were one tenth of that in plasma.

INTRODUCTION

Mammals subjected to environmental heat stress progressively alter their sympathetic-adrenomedullary and endocrine secretions. Epinephrine (E) and norepinephrine (NE) are two compounds secreted that are often used as indicators of the severity of such stress. Stress can come from exposure to cold or hot temperatures, psychological factors or adverse conditions such as during transport. It has been shown that livestock during transport show adverse physiological effects [1] and low feed consumption [2]. Additionally, significant increase in cortisol levels in calves' blood are also reported [3,4] during the marketing and shipping of calves.

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Plasma catecholamines (CAs) have been analyzed in cattle under conditions of environmental heat stress [5]. They showed a doubling of the concentration of plasma E during these preliminary studies. Davis *et al.* [6] have monitored the concentration of biogenic amines and have observed dramatic increases in their concentration during stress.

NE has been reported to be present in bovine milk, however, at very low levels [7,8]. Owing to the tedious nature of the assays there are only a few applications, particularly with cows, in this area.

The number of studies on the secretion of CAs during heat stress on lactating cows is limited owing to the need to couple controlled environmental conditions and a quantitative assay.

There are several assays available, traditional methods based on radiometric [9], radiometric–enzymic [10] and radioimmunoassay techniques [11]. However, reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) proves to be a reproducible and efficient method, which is suitable for clinical analysis [12,13]. This technique has been extensively reviewed [14–16] and is used in this study because of its sensitivity, versatility, speed and ease. Moreover, it avoids exposure to harsh organic solvents and radioactivity.

This paper reports the concentrations of NE and E in bovine plasma and milk, determined under conditions of controlled environmental heat stress. The response of the cows is also reported.

EXPERIMENTAL

Animals

Five lactating Holstein cows were exposed to three environmental temperature exposure periods. The experiment was conducted in the Missouri Animal Climatic Laboratory equipped with automatic controls programmed for temperature, humidity and light. Environmental conditions were controlled by first maintaining the chamber under thermoneutral conditions (TN_1), 18°C, 50% relative humidity (rh), for three days. This was followed three days of exposure to heat (H) at 36.6°C, 65% rh. Then the chamber was returned to thermoneutral conditions (TN_2) for a recovery period of three days at 18°C, 50% rh.

The changes in the chamber conditions from one period to the next were always made at 16:00 h. The animals were fed *ad libitum* three times daily at 07:00, 13:00 and 17:30 h. The ration was formulated on the basis of NRC requirements determined by the animal's body weight and level of milk production. Propionic acid (1% by weight) was added to the ration to preserve the quality of the feed for a one-week period. Plasma samples were taken at 07:00, 15:00 and 23:00 h each day by venipuncture of the tail vein using vacutainer tubes. The energy metabolism of each cow was measured during the course of the experiment. An indirect mask calorimetry apparatus, described by Baeta *et al.* [17], was used to measure O_2, CO_2 and CH_4 concentrations.

Plasma

The methodology for E determination was a modification of the procedure of Schadt and Gaddis [18]. A 2-ml volume of plasma was deproteinized by addition of 200 μl of 1 *M* perchloric acid (Fisher Scientific, St. Louis, MO, U.S.A.). Samples were then centrifuged at 12 000 *g* for 10 min at 4°C (Sorvall Model 2B centrifuge, Du Pont, Wilmington, DE, U.S.A.).

Milk

E was sampled in polypropylene tubes (VWR Scientific, Chicago, IL, U.S.A.). A 500-ml volume of 1 *M* perchloric acid and 5 ml of freshly collected milk were mixed by inverting the tubes several times and then placed on ice during the collection period. All samples were vortex-mixed in the laboratory and centrifuged at 12 000 *g* for 15 min. A 2-ml volume of clear supernatant was aspirated and stored in tubes at −70°C until extraction and HPLC–ED analysis.

Extraction procedure for epinephrine and norepinephrine

A 2-ml volume of the plasma or milk supernatant was placed in a 5-ml conical reaction vial along with 50 mg of acid-washed alumina (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The pH of each plasma or milk sample was adjusted to 8.6 by addition of 2 ml of Tris buffer with 5% ethylenediaminetetraacetate (EDTA) and 25 μl of 3,4-dihydroxybenzylamine (DHBA). The Tris buffer, EDTA and DHBA were purchased from Sigma (St. Louis, MO, U.S.A.). Each vial was vortex-mixed for 10 s and then shaken for 15 min in a reciprocal shaker (Fisher Scientific). After shaking, the supernatant was aspirated carefully to avoid the loss of alumina, washed three times with 3 ml of deionized water and transferred to 12.5 cm × 1.25 cm I.D. microfilter centrifuge tubes (MF-5500; Bioanalytical Systems) with 500 μl of deionized water, and centrifuged at 400 *g* for 4 min. The CAs were desorbed from the alumina, following the addition of 300 μl of 0.1 *M* perchloric acid, vortex-mixed and centrifuged at 400 *g* for 4 min. This extract was saved for injection in the HPLC system.

Method

An HPLC system with an electrochemical detector (Perkin Elmer, Norwalk, CT, U.S.A.) and amperometric control (Bioanalytical Systems) was used to measure the concentration of CAs [19]. A Beckman Model 114 M pump (St. Louis, MO, U.S.A.) was connected to a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector, equipped with a 200-μl fixed loop and a 250 mm × 4.6 mm I.D. Biophase C_{18} column packed with 5-μm particles (Bioanalytical Systems). The mobile phase was 2.5% (v/v) methanol–water. The aqueous solution contained 8.2 g/l anhydrous sodium acetate, 6.84 g/l citric acid, 324.3 mg/l octanesulphonic acid and 375 mg/ml Na_2EDTA. The mobile phase was filtered through 0.45-μm disks (Gilson, Middleton, WI, U.S.A.) and pumped at a flow-rate of 1.0 ml/min. The amperometer was set at a positive potential of +0.60 V with respect to the

Ag/AgCl reference electrode, with a sensitivity of 0.5 nA for the plasma samples. The acidic extract (0.2 ml) of the plasma or milk samples was injected into the HPLC–ED system to separate NE, E and DHBA (internal standard). For the milk samples the sensitivity setting was 0.25 nA, owing to the lower concentration in the milk. Occasionally, it was necessary to use the 0.5 setting for milk samples that had higher concentrations.

RESULTS AND DISCUSSION

The calibration curves were determined each day with standards injected just before and just after analysis of the unknowns. Calibration of the samples was made on the basis of peak heights, and a linear relationship was found in the region of interest. The r^2 values for NE, E and DHBA were 0.999, 0.999 and 0.998, respectively. Fig. 1 illustrates a typical chromatogram of the calibration standards using the method developed and described above. Retention times were 11 min for NE, 18 min for E and 26 min for DHBA.

Recoveries of CAs extracted from plasma and milk were 80.0 and 90.0%, respectively. Inter- and intra-assay variations for plasma NE were 6.58 and 3.85%, respectively, and for plasma E, they were 11.8 and 3.85%, respectively.

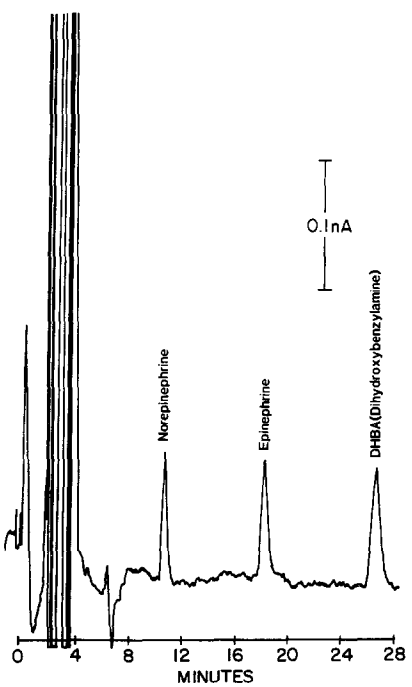

Fig. 1. Chromatogram of catecholamine standards containing norepinephrine (75 ng/ml), epinephrine (100 ng/ml) and DHBA (100 ng/ml).

Inter- and intra-assay variations for milk NE were 12.1 and 6.2%, respectively. Inter- and intra-assay variations for milk E were 8.6 and 9.7%, respectively.

Plasma samples taken under thermoneutral conditions were spiked with DHBA and analyzed by HPLC. A typical chromatogram is shown in Fig. 2. Under heat stress, the amounts of NE and E can be measured quantitatively by HPLC. Fig. 3 shows a typical chromatogram obtained under conditions of heat stress (H).

Similarly, the amounts of NE and E can be also determined quantitatively in the picogram range from the milk extract, using DHBA as an internal standard. Fig. 4 shows the chromatogram of the milk extract without stress and Fig. 5 during the first 16 h of stress. NE, E, DHBA and the unknown peaks were well resolved. The peak height of E is larger during heat stress compared with the thermoneutral period. Chromatograms such as those in Figs. 2–5 were obtained by analysis of the samples obtained from the animals as they were subjected to the controlled environment experiments.

The statistical analysis of the results for the different cows was accomplished using the Fisher's least significant difference (LSD) means method. The statistical

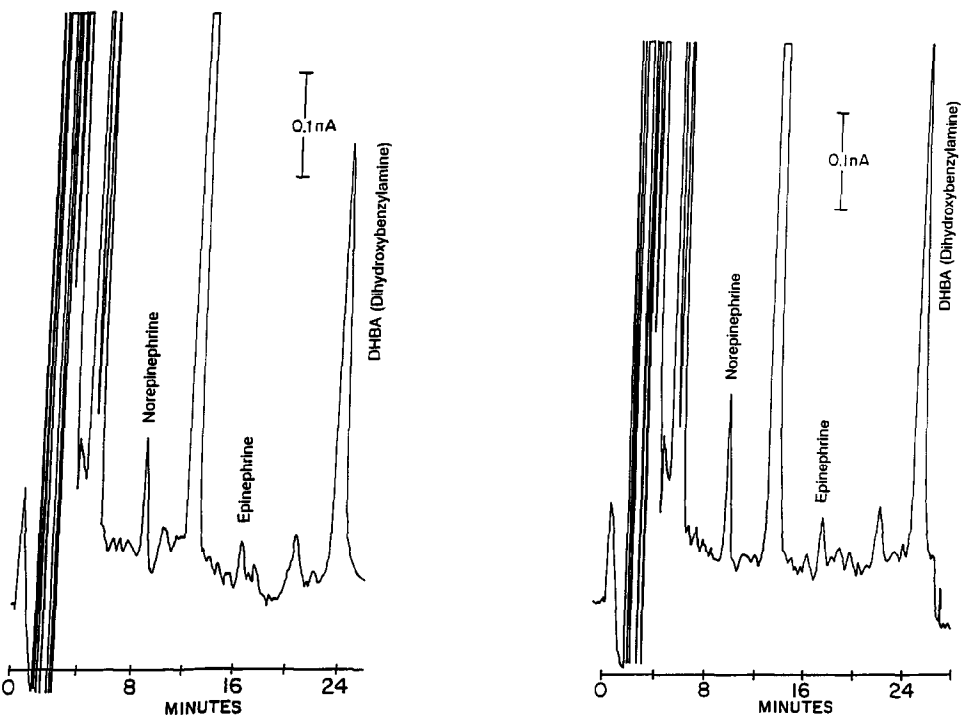

Fig. 2. Bovine plasma chromatogram during thermoneutral period; sensitivity, 0.5 nA.

Fig. 3. Bovine plasma chromatogram during the first 16 h of stress; sensitivity, 0.5 nA.

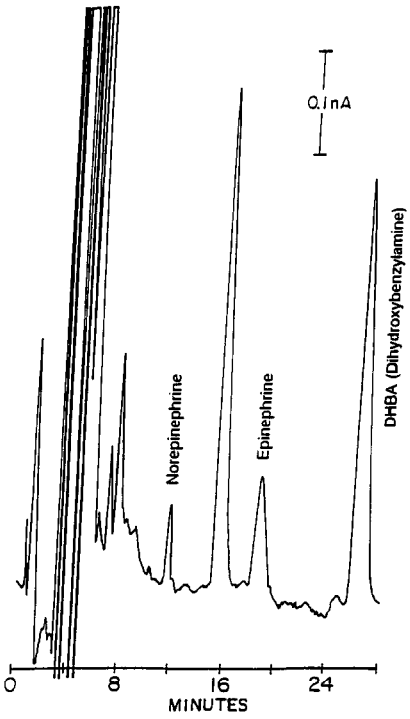

Fig. 4. Bovine milk chromatogram during thermoneutral period; sensitivity, 0.2 nA.

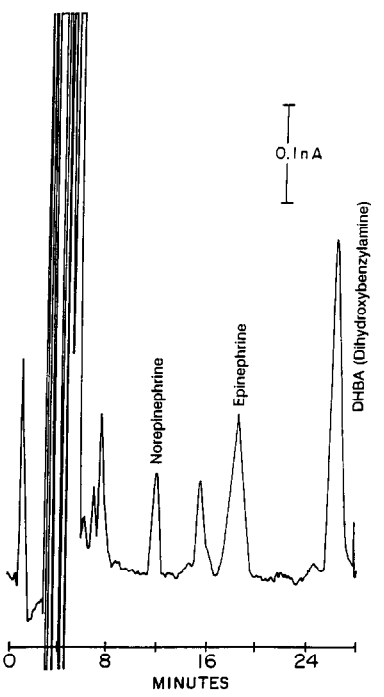

Fig. 5. Bovine milk chromatogram during the first 16 h of stress; sensitivity, 0.2 nA.

significance of various calculated mean concentrations was determined for the H and the TN_2 periods compared with the TN_1 period. The results are summarized in Tables I–III.

Table I presents the average values of the plasma NE and E concentrations and the rectal temperatures during the thermoneutral period (TN_1), the first 32 h

TABLE I

EFFECT OF HEAT EXPOSURE ON PLASMA CATECHOLAMINES

Period	Concentration (mean ± S.D.) (pg/ml)		Rectal temperature (mean ± S.D.) (°C)	E/NE ratio
	NE	E		
TN_1 (0–24 h)	119.7 ± 10.9	52.5 ± 5.1	38.3 ± 0.19	0.44
H_1 (0–32 h)	199.3 ± 17.3[a]	142.5 ± 4.9[a]	39.8 ± 0.16[a]	0.72
H_2 (40–72 h)	111.5 ± 11.9	50.3 ± 9.9	40.8 ± 0.25[a]	0.45
TN_2 (0–72 h)	122.4 ± 11.6	73.8 ± 12.9	38.6 ± 0.09	0.6

[a] The calculated number is statistically significantly different from the TN_1 period.

TABLE II

EFFECT OF HEAT EXPOSURE ON MILK CATECHOLAMINES

Period	Concentration (mean ± S.D.) (pg/ml)		E/NE ratio
	NE	E	
TN_1 (0–24 h)	17.2 ± 2.6	48.9 ± 9.3	2.84
H_1 (0–32 h)	15.9 ± 1.67	193.5 ± 10.9[a]	12.2
H_2 (40–72 h)	30.4 ± 6.2[a]	110.6 ± 27.8[a]	3.64
TN_2 (0–72 h)	26.6 ± 6.4	79.6 ± 4.8	3.0

[a] The calculated number is statistically significant from the TN_1 period.

of heat exposure (H_1), the second 40 to 72 h exposure period (H_2) and the post-thermoneutral period (TN_2). The average NE levels increased significantly during the first 32 h period of heat exposure. During the last 40 to 72 h of heat exposure the concentration returned to a value that is not significantly different from the TN_1 period. During the TN_2 period the NE levels were not statistically different from the TN_1 period.

Similarly, the plasma E concentration (Table I) also increased, relative to the TN_1 period, during the first 32 h of heat (H_1). During H_2, the level returned to values that are not statistically different from the TN_1 period. During the second thermoneutral period, TN_2, the plasma E concentrations were the same as in the TN_1 period. On the other hand, the rectal temperature continued to rise during both H_1 and H_2.

Fig. 6 illustrates graphically the rectal temperature and the plasma NE and E concentrations as a function of the time that the cows were subjected to heat

TABLE III

PHYSIOLOGICAL PARAMETERS DURING STRESS PERIODS

Values are means ± S.D.

Period	Milk production[a] (kg/milking)	Feed intake (kg/24 h)	Energy metabolism (kJ/h)	Water intake (l/24 h)	Rectal temperature (°C)
TN_1 (0–24 h)	12.97 ± 0.78	40.42 ± 1.61	4179.0 ± 255.1	56.20 ± 4.6	38.33 ± 0.19
H_1 (0–32 h)	12.45 ± 0.86	30.40 ± 6.82[b]	3914.6 ± 263.0	81.48 ± 14.2[b]	39.83 ± 0.16[b]
H_2 (40–72 h)	8.68 ± 0.81[b]	23.55 ± 1.25[b]	3025.1 ± 226.0[b]	63.86 ± 13.2[b]	40.82 ± 0.25[b]
TN_2 (0–72 h)	11.97 ± 0.96	36.10 ± 2.50	4067.0 ± 165.0	57.31 ± 4.42	38.59 ± 0.09

[a] Milk was taken twice per day and averaged.

[b] The calculated number is statistically significant from the TN_1 period.

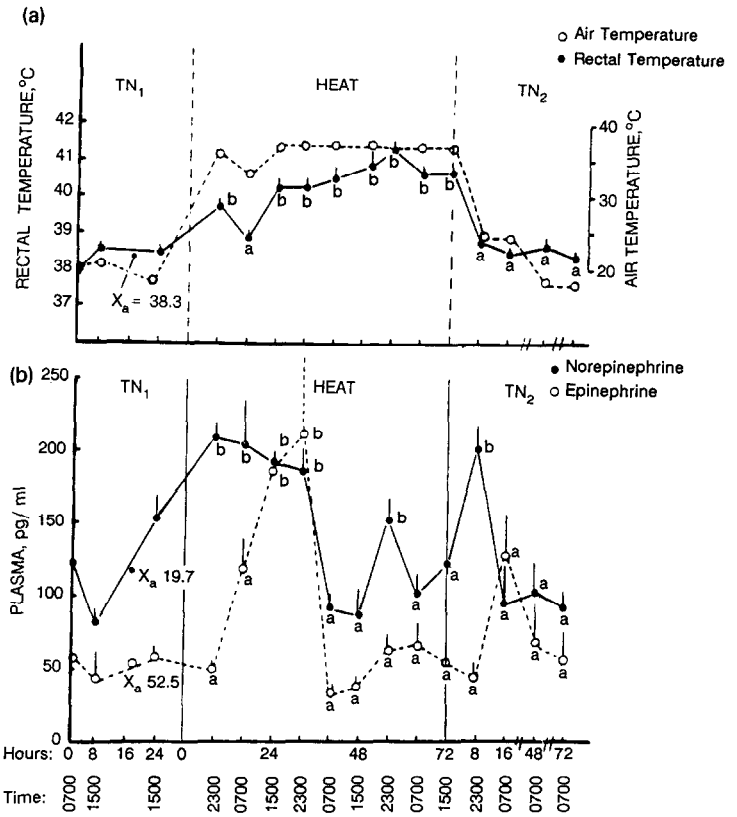

Fig. 6. Effect of environmental temperature on the catacholamine concentration and the rectal temperature. (a) Rectal temperature; (b) plasma epinephrine and norepinephrine concentration. Values marked a are not statistically significant from the mean value calculated during TN_1. Values maked b are statistically significant from the mean value calculated during TN_1.

stress. The values of time on the x-axis are expressed both in hours and in time of day. Fig. 6a shows the rectal temperature and air temperature during TN_1, heat and TN_2 periods. The rectal temperatures closely followed the air temperature and remained elevated during the entire 72 h of heat exposure. In Fig. 6b, the NE concentration increased significantly in the first 24 h, remained elevated for 32 h of heat exposure, and then declined (with the exception of 56-h samples) during the 40–72 h heat period. The increase in E was delayed, only attaining significant levels by 32 h. It then declined and remained at the TN levels for the remainder of the heat period.

Table II presents the effect of heat exposure on the milk CAs. In contrast to the trend for the plasma NE values, the average concentration for the milk samples remained constant during the TN_1 period and during the first 32 h of heat (H_1). During the last 40–72 h (H_2) of heat there was an increase in the concentration of

NE. Upon returning to thermoneutral conditions, the concentration of NE returned to a value that is not statistically significantly different from the TN_1 state. The concentration of E in milk, however, increased dramatically during the H_1 period, reduced during the H_2 period and then reduced further during the TN_2 period.

Tables I and II also show the ratio of E to NE for plasma and milk, respectively. For example, in plasma, exposure to heat (H_1) results in a decrease in the adrenomedullary release of E relative to NE, whereas in milk, the concentrations of E were much greater than those of NE.

Table III summarized the milk yields, feed intake, energy metabolism, water intake and rectal temperature during the different periods of the experiment. Milk production and energy metabolism remained constant durign the H_1 period, but decreased significantly during the H_2 period. Feed intake decreased during H_1 and H_2. Water intake increased markedly during H_1 and the rectal temperature increased steadily during both H_1 and H_2. All parameters recovered to normal during the TN_2 period.

CONCLUSIONS

The effect of heat stress lead to significant differences in the concentrations of E and NE in bovine plasma and milk. These differences will help to ascertain the mechanism by which these catecholamines are excreted from the animal [20].

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