PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 420–423 (1979)

Deleterious Effects of Leucine Administration in Endotoxin Shock (40695)

ROY D. GOLDFARB,[1] PETER WEBER, JUNE EISENMAN, AND DANIEL J. LOEGERING

Departments of Physiology and Biochemistry, Albany Medical College, Albany, New York 12208

The concept that the elaboration of circulating toxic substances during shock exacerbates the shock state has been proposed by numerous authors (1–3). Recently, we reported the isolation of one substance which exerted cardiodepression (1). We isolated (via gel filtration and cation-exchange chromatography) this substance from plasma obtained from dogs in irreversible hemorrhagic shock which we identified as L-leucine. These findings led to the following hypothesis: If leucine is a circulating toxic shock substance and if it is important in the pathogenesis of irreversible shock, then the blood levels of leucine at the time of death should be elevated in shocked animals and administration of leucine to shocked animals should hasten the onset of cardiovascular failure. In order to directly test this hypothesis, we subjected male, Sprague–Dawley rats to endotoxin shock and administered leucine, while monitoring survival times and circulating leucine levels.

Methods. a. Endotoxin shock. Two groups of male rats (350–375 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The femoral artery was cannulated and 1.0 mg/100 g *Salmonella enteritidis* endotoxin (Difco, Batch 649204) was injected over 1 min. Pilot studies revealed that this dose of endotoxin was approximately an LD_{90}. Immediately after endotoxin injection, either 0.153 *M* leucine or isoleucine was infused at a dose of 7.6 mmole/kg, or the equivalent volume of Krebs–Henseleit vehicle was infused through the femoral artery catheter. These solutions were infused at 0.33 ml/min and took approximately 35 min for each animal. This long infusion time and high infusion volume was necessitated by the low water solubility of leucine. In the first group, each animal had three blood samples (0.3 ml each) drawn at the following times: (i) prior to endotoxin infusion; (ii) after amino acid or vehicle infusion; and (iii) at death. After endotoxin and either leucine, isoleucine, or KH infusion, each animal was observed and the time between the end of infusion and death (defined as the spontaneous cessation of heart beat via manual palpitation) was recorded. Infusion of 7.6 mmole/kg leucine into nonshocked rats was also performed. In the second group, blood samples (2 ml) were taken at ½, 2, or 3 hr after the end of leucine or KH infusion, and the animal was terminated.

b. Blood sample analysis. The collected blood samples from each animal were allowed to clot and centrifuged and the serum was collected. Each serum sample was deproteinized by the mixing of 100 μl 10% trichloroacetic acid containing 12.5 nmole of norleucine as an internal standard to 100 μl serum. After centrifugation, 100 μl of deproteinized serum was applied to an Aminex A-4 cation-exchange column (0.9 × 50 cm) and eluted with Pico buffer B (Pierce Chemical Co.) at 50° and a flow rate of 60 ml/hr. In this system, all of the acidic amino acids elute with the buffer front and methionine, isoleucine, leucine, and norleucine elute at 51, 58, 64, and 70 min, respectively. For the detection system we used an automated orthophthalaldyhyde (OPA) reaction system (4) where the reagent consisted of 0.08% OPA in 0.4 *M* potassium borate, pH 10.4, which was mixed by pumping at 48 ml/hr with the column effluent in a 0.5-mm-i.d. × 3-m-length Teflon coil. Fluorescence was measured in a continuous-flow fluorometer (Laboratory Data Control) and recorded at 6 in./hr on a 10-in. strip chart recorder. Peak integration was performed by manual triangulation and the data were expressed in μ*M* concentration. After each analysis the column was regenerated for 15 min with 0.7 *N* NaOH containing 0.2% w/v ethylenediamine tetraacetic acid

[1] To whom all correspondence and reprint requests should be addressed

0037-9727/79/110420-04$01.00/0

tetrasodium salt and 0.2% v/v of a 30% Brij 35 (Atlas Chem. Ind.) solution in order to prevent loss of resolution due to peptide absorption on the ion-exchange resin.

c. Data analysis. The difference between the time of death for leucine, isoleucine- and KH-administered rats and the serum amino acid concentrations at analogous times were compared using the Student's *t* test for unpaired data to determine significance. The changes in serum amino acid concentrations in each group at sequential times were compared using the paired Student's *t* test. Correlation of the time to death and each serum amino acid concentration was performed using linear regression analysis. Significance in all tests were assumed to be reached at $P < 0.05$.

Results. The data derived from the first group of endotoxin-shocked rats is presented in Table I. The infusion of leucine into endotoxin-shocked rats ($N = 8$) accelerated the shock syndrome as evidenced by the significantly lower time to death in the leucine animals ($P < 0.01$ compared to KH-infused shocked rats) ($N = 9$). The isoleucine-administered shock rats ($N = 9$) tended to expire sooner than the KH-infused shock rats ($P = 0.06$). Infusion of leucine or isoleucine at 7.6 mmole/kg body wt concentrations in nonshocked rats had no apparent effect, i.e., these animals were 24-hr survivors. Measuring the serum concentration of leucine and isoleucine revealed that after endotoxin administration and KH infusion, both leucine and isoleucine concentrations were elevated at death ($N = 9$) (leucine: from 140 ± 9 to 351 ± 25 μM, ($P < 0.01$); isoleucine: 104 ± 6 to 176 ± 4 μM ($P < 0.01$), mean ± SEM). In the isoleucine-infused animals the serum isoleucine concentrations at the time of death were significantly elevated ($P < 0.01$). However, in these animals, serum leucine was also elevated compared to KH-infused shocked rats at time of death (507 ± 27 vs 351 ± 25 μM, respectively, $P < 0.05$). In contrast, the isoleucine concentrations at the time of death in the leucine-administered animals were not different from the isoleucine concentration in shocked rats given KH (176 ± 4 vs 174 ± 18 μM, respectively). The serum leucine concentrations at the time of death in the leucine-infused rats were significantly higher than both other groups ($P < 0.01$).

The terminal serum leucine concentrations were elevated in all groups compared to preshock levels. There was an inverse correlation between blood leucine concentration at time of death and time to death ($r = 0.415$, $P = 0.02$). The data appears to be curvilinear rather than linear which may account for the low correlation coefficient. The observations that terminal serum leucine concentrations in the isoleucine-infused, endotoxin-shocked animals were elevated compared to those of KH-infused rats and that the isoleucine-infused animals also died earlier than the KH-infused rats was unanticipated.

The serum leucine concentration of the second group of rats is presented in Table II. Following KH infusion in the sham-shocked rats, leucine levels did not change significantly. Following leucine infusion in the sham rats, serum leucine concentrations were markedly elevated and they remained so through the 3-hr observation. The KH-infused endotoxin shock group demonstrated a significant transient elevation in serum leucine at ½ hr. The endotoxin-shocked, leucine-infused rats had a significantly higher serum

TABLE I. SERUM LEUCINE AND ISOLEUCINE CONCENTRATIONS FOLLOWING ENDOTOXIN ADMINISTRATION

Infusate	*N*	Time to death (min)	Leucine (μM)			Isoleucine (μM)		
			A	B	C	A	B	C
Krebs Henseleit	9	274 ± 24	140 ± 9	212 ± 10[a]	351 ± 25[a]	104 ± 6	122 ± 3	176 ± 4[a]
Isoleucine	9	201 ± 26[b]	145 ± 7	[c]	507 ± 27[a, d]	104 ± 4	14075 ± 1289	2020 ± 824[a, d]
Leucine	8	185 ± 10[d]	139 ± 8	11343 ± 1138	839 ± 89[a, d]	102 ± 4	—[e]	174 ± 18[a]

[a] Data reported as mean ± 1 SEM. Sample A prior to endotoxin, B after infusion, C at death. [a] $P < 0.01$ compared to sample A, paired *t* test.

[b] $P = 0.06$ compared to KH, group *t* test.

[c] Not measurable due to high isoleucine concentration.

[d] $P < 0.05$ compared to KH, group *t* test.

[e] Not measurable due to high leucine concentration.

TABLE II. SERUM LEUCINE CONCENTRATION FOLLOWING ENDOTOXIN OR VEHICLE INJECTION AND LEUCINE OR KREBS–HENSELEIT INFUSION

Group	½ hr	2 hr	3 hr
Sham–KH	190 ± 30 (5)	158 ± 12 (5)	165 ± 5 (5)
Sham–Leu	4973 ± 226 (4)	842 ± 101 (5)	456 ± 57 (6)
Endo–KH	336 ± 31 (6)	134 ± 7 (5)	152 ± 11 (6)
Endo–Leu	6589 ± 167[a] (6)	1510 ± 270[b]	843 ± 176[b] (5)

[a] All values ± SE (N) μM leucine. Time after end of leucine or KH infusion. Value prior to endotoxin or sham injection is 142 ± 5 M. $P < .01$ compared to Sham–leu.

[b] $P < .05$ compared to Sham–leu.

[c] $P < 0.01$ compared to Sham–KH.

leucine concentration at all times following infusion than did the sham-shocked, leucine-infused rats indicating that endotoxin shock inhibited the catabolism of the exogenous leucine.

Discussion. The experiments reported in this study were based on our observations that leucine may be a circulating toxic shock substance (1, 3). We tested the hypothesis that if leucine was a toxic shock substance, then the exogenous administration of leucine to a shocked animal should accelerate the shock syndrome and serum leucine concentrations should be elevated in the vehicle-infused shocked animals. The dose of leucine (or isoleucine, used as an amino acid control) chosen for infusion was calculated to raise serum leucine at the end of infusion to approximately 7.6 mM, a level which exerted significant depressant activity in the feline right ventricular papillary muscle bioassay as previously reported (1, 3). We rationalized that if it did not accelerate the shock syndrome at this high concentration, then it probably would not have any *in vivo* significance. Cardiac function was not measured in these animals because of a complex interpretive problem. *A priori,* since all of these animals will exhibit cardiac failure after endotoxin administration (LD_{90}), it would be impossible to determine if the rate of the development of cardiac hypodynamics was faster than the rate of the development of cardiovascular collapse in the leucine-infused animals compared to the KH-infused controls.

The findings of this study indicate that elevation of serum leucine may be a contributing factor in the development of the shock syndrome. The administration of these large amounts of exogenous leucine did significantly accelerate the endotoxin shock syndrome. An elevation of serum leucine by a factor of 2.5 in the KH-infused, endotoxin-shocked rats indicated that leucine may indeed exert a pathophysiological action *in vivo.* Although the exact mechanism for the shock-accelerating action of leucine was not approached in this study, the observation that leucine did not induce shock in nonshocked animals suggests that leucine acts synergistically with another shock product and/or the shock syndrome induces a dysfunction of leucine metabolism allowing for its serum accumulation. A specific shock-accelerating role for leucine was suggested by observation of elevated sera leucine concentrations in the isoleucine-infused group which was associated with an earlier time of death for these animals. Since the converse (elevated isoleucine levels in the leucine-infused rats) was not observed, this suggests a specific role for leucine rather than a nonspecific effect of amino acids. The apparent shock-accelerating action of leucine may be magnified by a dysfunction of leucine catabolism following endotoxin. We observed significantly higher serum leucine concentrations after leucine infusion in the endotoxin-shocked rats than in the sham animals.

Previous investigations have reported similar plasma or serum elevations in leucine concentrations following traumatic injury or clinical shock episodes (5, 6). Levenson *et al.* (5) reported that plasma leucine levels do indeed rise dramatically during the initial response to traumatic injury caused by battle casualties. They reported increases in the range of two to five times control values; the concentrations reported were in the concentration range which we found to be cardiodepressant *in vitro* (3). Border *et al.* (6) have also reported elevated plasma leucine levels of traumatically injured patients. They reported that leucine, along with many other amino acids, ketones, and triglycerides begin at a low level, reach maximum values at 5–10 days, and drop by 21 days in patients successfully treated for multiple organ system failure induced by traumatic injury via gunshot wound, automobile, or industrial accidents.

Summary. We have recently reported the isolation and identification of leucine from the plasma of shocked animals which exerted significant cardiodepressant activity *in vitro.* This study tested the hypothesis that if leucine is a circulating-factor *in vivo* and if this has any significant deleterious effects in the shocked animal, then the exogenous administration of leucine should accelerate the shock syndrome. We infused leucine, isoleucine (7.6 mmole/kg), or KH into endotoxin-shocked rats and measured survival times and blood concentrations of leucine and isoleucine. Following endotoxin administration, the leucine-administered rats died significantly sooner ($P < 0.01$) than the KH-infused animals; the isoleucine animals also died sooner ($P = 0.06$). Terminal leucine concentrations were elevated in all animals following endotoxin administration compared to preshock concentrations. The terminal serum leucine concentration in the leucine- and isoleucine-infused groups was significantly higher ($P < 0.06$) than the KH-infused group. In contrast, terminal serum isoleucine concentrations were significantly elevated only in the isoleucine-infused group. These data suggest that the elevated serum leucine concentration may contribute to the development of irreversible shock.

This research was supported by Grant HL 19977 from the National Institutes of Health. Dr. Goldfarb is the recipient of Career Research Development Award HL-00947. We thank Mrs. Joanne Bayreuther for her assistance in the preparation of this manuscript.

1. Goldfarb, R. D., Weber, P., and Estes, J. E., Fed. Proc. **37,** 2724 (1978).
2. Lefer, A. M., Circ. Res. **32,** 129 (1973).
3. Goldfarb, R. D., Weber, P., and Eisenman, J., Amer. J. Physiol. **237,** H168 (1979).
4. Benson, J. R., and Hare, P. E., Proc. Nat. Acad. Sci. USA **72,** 619 (1975).
5. Levenson, S. M., and Rosen, H., Surg. Gynecol. Obst. **101,** 35 (1955).
6. Border, J. R., Chenier, R., McMenany, R. H., LaDuca, J., Seibel, R., Birkhan, R., and Yu, L., Surg. Clin. North Amer. **56,** 1147 (1976).
7. Goldfarb, R. D., Circ. Shock Suppl. **1,** 23 (1979).
8. Lefer, A. M., Fed. Proc. **37,** 2739 (1978).

Received January 11, 1979. P.S.E.B.M. 1979, Vol. 162.