Effect of Insulin on Creatinuria and Hypercreatinemia Induced by Creatine Loading[1] (35168)

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Much has been written about the effect of insulin on the transmembrane transport of glucose and amino acids, on protein synthesis, and on the movement of various inorganic ions into and out of intracellular and extracellular compartments (1–4). Although several observations suggest that insulin may also influence the metabolism of creatine and phosphorylcreatine (5, 6), this area of investigation has received relatively little attention. Recently, we observed that radiation-induced creatinuria can be prevented by insulin (7). The mechanism by which insulin produces this effect is not yet clear, although several hypotheses have been proposed to explain the action of the hormone in suppressing creatinuria in X-irradiated rats (7). In view of the possibility that insulin may influence creatine metabolism in normal as well as X-irradiated animals, it appeared desirable to study the effect of insulin on the creatine content of blood and urine in rats which were made creatinuric by the intraperitoneal (ip) injection of creatine. The results of the present studies showed that creatinuria induced by creatine loading can be prevented in the rat by the prior administration of insulin. Furthermore, the hypercreatinemia which was observed shortly after the ip injection of a creatine load was suppressed by insulin, suggesting that the action of the hormone in preventing creatinuria is related to its effect on the blood creatine concentration rather than to an effect on the kidneys *per se*.

Materials and Methods. Male rats of the Wistar strain, weighing 165 ± 8 g were caged individually in metabolism cages. The rats fed freely on Purina Laboratory Chow and were given water to drink. Urine was collected every 24 hr, filtered, and then was frozen and stored at −15°. One group of rats was injected subcutaneously (sc) daily with 0.5–2.0 units of NPH insulin (Squibb Co.) and a second group was injected with 0.1 ml of physiological saline. On the second day of treatment with insulin, all rats were injected ip with 5–10 mg of creatine dissolved in 1 ml of Ringer's solution. Creatine was given in the morning approximately 1 hr after the injection of insulin. Urine was collected for 1 day before and for 1 or 2 days after the administration of creatine. Urinary creatine was determined by a micromodification of Kibrick's method (8) which is based on the coupled enzyme procedure described by Tanzer and Gilvarg (9). The details of the method differ from the original one in several respects and are described as follows.

Two ml of rat urine were adjusted to pH 9.0 by the dropwise addition of 6% NaOH and the sample was made up to 5.0 ml with 0.15 *M* sodium glycinate buffer, pH 9.0. Two-tenths ml of the clear, diluted urine was added to 1 ml of a modified Kibrick's reagent No. 6 (8). This reagent was composed of a mixture of 10 ml of reagent No. 1 (0.083 *M* sodium glycinate buffer, pH 9.0); 0.5 ml of reagent No. 2 (0.01 *M* magnesium chloride); 0.25 ml of reagent No. 3 (69.2 mg of adenosine-5′-triphosphate, disodium trihydrate, Boehringer-Mannheim Co., dissolved in 5 ml of H_2O); 0.5 ml of reagent No. 4 (30 mg of diphosphopyridine nucleotide, reduced form, Sigma Co., dissolved in 10 ml of H_2O); 0.25 ml of reagent No. 5 (60 mg of 2-phosphoenolpyruvic acid, cryst., monosodium monohy-

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TABLE I. Urinary Creatine Excretion in Rats Injected Intraperitoneally with Creatine.[g]

Treatment	Creatine (mg/24 hr); days after injection of creatine		
	−1	+1	+2
Creatine, 5 mg	0.501 ± 0.034 (13)	1.40 ± 0.07 (13)[a]	1.06 ± 0.05 (13)[c]
Creatine, 5 mg + insulin, 2 units daily	0.527 ± 0.040 (13)	0.606 ± 0.047 (13)[b]	0.458 ± 0.053 (13)[d]
0.5 units daily	0.313 ± 0.025 (11)	0.486 ± 0.090 (11)	—
Creatine, 10 mg	0.461 ± 0.050 (8)	2.88 ± 0.28 (8)[e]	0.583 ± 0.41 (8)
Creatine, 10 mg + insulin, 2 units daily	0.546 ± 0.068 (8)	3.66 ± 0.12 (8)[f]	0.516 ± 0.051 (8)
Insulin, 2 units daily	—	0.417 ± 0.050 (10)	0.389 ± 0.039 (10)

[a] Student t probability: a vs b, $p < .001$; c vs d, $p < .001$; e vs f, $p < .025$.

[g] Values are means ± SE, number of observations is given in parentheses.

drate, Boehringer-Manneheim Co., dissolved in 5 ml of H_2O); and 30 ml of H_2O and was prepared freshly each day. Ten μl of reagent No. 7 (a mixture of 0.25 ml of lactic dehydrogenase [LDH], 5 mg/ml; 0.30 ml of phosphoenol pyruvate kinase [PK], 10 mg/ml, Boehringer-Mannheim Co.; and 1.62 ml of H_2O) were added to the test system with mixing and the optical density (OD) of the mixture was determined after 12 min at room temperature in a Zeiss PMQII spectrophotometer at 340 mμ using water as a reference solution. A reagent blank containing the complete mixture minus the urine sample was made up to volume with water and its OD also was determined 12 min after the addition of LDH-PK. Fifty μl of reagent No. 9 (6.7 mg of creatine phosphokinase [CPK], Boehringer-Mannheim Co., dissolved in 1 ml of 0.01 *M* sodium glycinate buffer, pH 9.0) was then added to both the experimental and control tubes with mixing and the OD of the solutions was again determined after 30 min. Standards containing 5 and 10 μg of creatine were always included along with the experimental samples. The concentration of creatine in urine was calculated from the decrease in OD of the experimental sample corrected for the change in OD of the reagent blank after addition of CPK.

In another experiment, male rats weighing 176 ± 9 g were injected sc with 2 units of NPH insulin or with 0.1 ml of physiological saline. About 1 and 1.5 hr after treatment with insulin the animals were lightly anesthetized by the sc injection of pentobarbital (5 mg/100 g of body wt). In some cases, the anesthetized rats awoke (usually after 1–1.5 hr) during the course of the experiment and these were given an additional 1–2 mg of pentobarbital sc. Two hr after the injection of insulin, the rats were injected ip either with 5 mg of creatine dissolved in 1 ml of Ringer's solution or with Ringer's solution alone. The tail of each anesthetized rat was transected at its tip and 0.1 ml of blood was collected in a heparinized capillary tube just prior to and immediately after the ip injection of creatine. The samples of blood were mixed with 0.3-ml aliquots of $Ba(OH)_2$ and $ZnSO_4$ reagents as described by Van Pilsum *et al.* (10) and the mixtures were allowed to stand for 1–2 hr. One-tenth-ml samples of blood were collected from the tail every 15 min for 2 hr after injection of creatine and were treated with $Ba(OH)_2$ and $ZnSO_4$ as described. Loss of blood was minimized by taping the terminal 2–3 cm of the tail to a support in such a way that the tail remained in an uptright position between bleedings. After centrifugation at 2500*g* for 15 min, the deproteinized samples of blood were assayed for creatine by the α-naphthol micromethod essentially as described by Gerber *et al.* (11).

Results. As shown in Table I, male rats injected with 5 mg of creatine exhibited creatinuria for 2 days immediately following the administration of creatine. Assuming that normal urinary creatine excretion in these

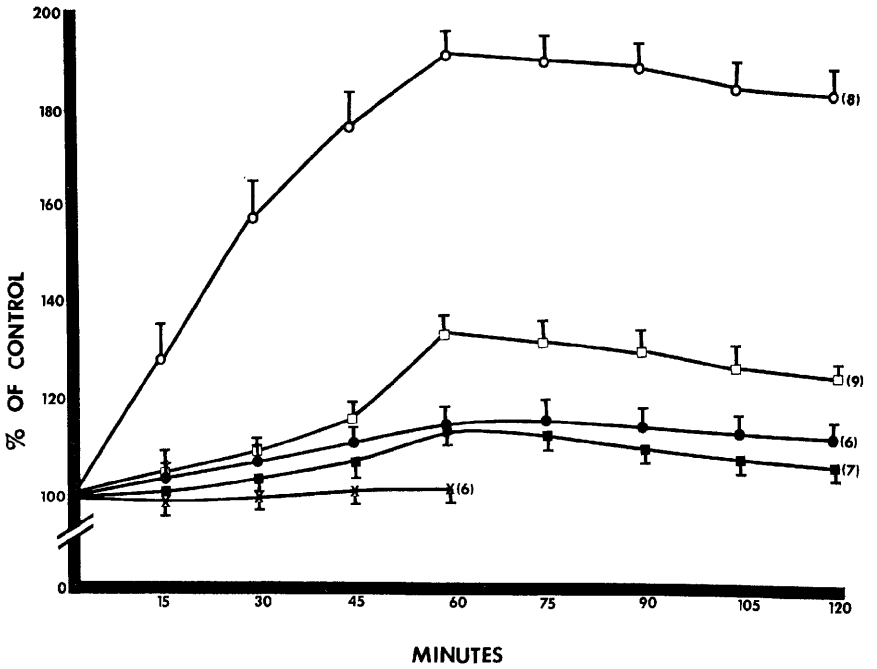

FIG. 1. Effect of an ip injection of creatine on the concentration of creatine in blood: Values shown are means ± SE expressed as percentage of controls for comparison. Number of observations is given in parentheses. Rats received the following treatment: (○), 5 mg of creatine, ip; (●), 5 mg of creatine, ip, plus 2 units of insulin, sc; (□), 1 ml of Ringer's solution, ip; (■), 1 ml of Ringer's solution, ip, plus 2 units of insulin, sc; and (x), no treatment. The concentration of creatine in the blood of each group of rats at time zero was 1.44 ± 0.08, 1.54 ± 0.08, 1.01 ± 0.09, 0.93 ± 0.07, and 1.21 ± 0.03 mg of creatine/100 ml of blood, respectively.

rats prior to the injection of creatine was about 0.5 mg of creatine/24 hr, approximately 30% of the dose was excreted in the urine after 48 hr. In contrast, rats treated with either 0.5 or 2 units of insulin before the injection of 5 mg of creatine, excreted creatine at levels which were not significantly different from those found before creatine loading. On the other hand, the prior injection of 0.25 units of insulin in animals given 5 mg of creatine did not consistently prevent creatinuria. Although the data have not been tabulated, it was observed that creatinuria could be prevented in about 50% of the animals with this dose of insulin. As might be expected, the group of rats injected ip with 10 mg of creatine exhibited a more intense creatinuria than those which had received 5 mg of creatine. In contrast to the findings with the insulin-treated rats given 5 mg of creatine, the prior injection of 2 units of insulin did not suppress creatinuria in animals injected with 10 mg of creatine. Furthermore, the rats which received 10 mg of creatine exhibited creatinuria only on the first day following the injection of creatine, while the rats which were given 5 mg of creatine excreted increased amounts of creatine on both days following the injection. In addition, the insulin-treated rats injected with 10 mg of creatine excreted *more* creatine than the untreated, creatine-injected animals. The reason for these differences between the two groups of rats is not known. As shown in Table I, the administration of insulin to control rats injected ip with 1 ml of Ringer's solution did not produce a significant change in the daily excretion of creatine when compared to that of untreated rats before creatine-loading.

Figure 1 shows the kinetics of the change in creatine concentration in the blood of rats after the ip injection of 5 mg of creatine. The average creatine concentration of blood prior

to the injection of creatine for 8 untreated rats was 1.44 ± 0.08 mg of creatine/100 ml of whole blood. The concentration of creatine in the peripheral blood increased to approximately 130% of the preinjection (control) level in 15 min after the injection of creatine and reached a maximum of 190% of the control value after 60 min. The creatine concentration in the blood then declined slowly reaching a value which was 180% of the control level in 2 hr after the initial injection of creatine. In contrast, the creatine concentration in the blood of rats treated with 2 units of insulin 2 hr prior to the injection of 5 mg of creatine increased only slightly 15 min after the injection of creatine and reached a maximum of 114% of the control value after 60 min. The creatine concentration in blood then decreased to a level which was about 109% of the control 2 hr after the injection of creatine. The average creatine concentration in blood prior to the ip injection of creatine for 6 insulin-treated rats was 1.54 ± 0.08 mg of creatine/100 ml of whole blood and was not significantly different from that of the untreated animals. The total blood lost by each animal during the experiment was approximately 1.1–1.3 ml and represented less than 10% of the estimated total blood volume of the rat.

Also shown in Fig. 1 are the kinetics of the change in creatine concentration of the blood in control rats treated with and without insulin and injected ip with 1 ml of Ringer's solution 2 hr after treatment with the hormone. Surprisingly, a moderate hypercreatinemia which was maximal 1 hr after injection of Ringer's solution was observed in the untreated rats. The average blood creatine concentration increased to 133% of the preinjection control level after 1 hr and fell to 122% of the control after 2 hr. The creatine concentration in the blood of rats treated with 2 units of insulin increased to 115% of the control level 1 hr after injection of Ringer's solution and decreased to 105% of the control after 2 hr. Thus, insulin not only suppressed the hypercreatinemia that occurred after creatine loading but also suppressed the moderate hypercreatinemia that occurred in otherwise "normal" rats, which served as controls and received no creatine. It was not clear from these data whether the hypercreatinemia observed in animals injected ip with Ringer's solution was related to the ip injection itself, the repeated blood sampling and consequent loss of blood, the anesthesia with pentobarbital, or to some combination of all 3 factors.

Accordingly, 6 rats were anesthetized and bled every 15 min for 1 hr exactly as described in the latter experiments, except that the animals were *not* injected ip with Ringer's solution. The results showed that the concentration of creatine in blood did not change after 1 hr in the untreated animals. These findings indicate that the ip injection of Ringer's solution was responsible for the moderate hypercreatinemia observed in control rats which were not injected with creatine. Although the reason for this effect is not clear, it appears from these studies that the creatine concentration of the blood is quite sensitive to experimental manipulation in the rat.

Discussion. The observations recorded here (Table I) along with our previous studies reported elsewhere (7) demonstrate that insulin can prevent creatinuria in two widely different conditions, namely, creatinuria induced by creatine loading and that induced by exposure to X-irradiation, the former condition being associated with the urinary excretion of exogenous creatine and the latter with the excretion of endogenous creatine. Furthermore, we have defined the minimum dose of insulin which will suppress load-induced creatinuria and the maximum creatine load beyond which creatinuria is no longer prevented by insulin in the rat.

The creatinuria observed in these studies occurs very likely as a direct result of hypercreatinemia which is produced shortly after the ip injection of creatine. Insulin could prevent load-induced creatinuria in one of three ways: (a) the hormone may suppress hypercreatinemia which occurs after creatine loading and thereby may prevent the concentration of creatine in blood from exceeding the kidney threshold for creatine; (b) insulin may alter the kidney threshold for creatine so that creatinuria will not occur despite the

presence of hypercreatinemia; or (c) insulin may give rise to excessive excretion of a metabolite which competes with creatine at the renal level and is excreted in preference to creatine, thus inhibiting creatine excretion. Since the blood creatine concentration did not increase substantially in creatine-injected animals treated with insulin as compared to the untreated controls, our results indicate that the first hypothesis (a) best explains the role of insulin in preventing creatinuria induced by creatine loading in the rat. However, the data do not shed any light on the details of the mechanism whereby insulin prevents load-induced hypercreatinemia and the associated creatinuria. Insulin could, for example, act to facilitate or enhance the transmembrane transport of creatine in skeletal muscle as it does in the case of glucose (1) and certain amino acids (2). Experiments now underway in our laboratory are designed to test this hypothesis by determining if insulin can influence the uptake of radioactive creatine by skeletal muscle in the intact animal *in vivo* and in isolated muscle preparations *in vitro*. It is of interest that a preliminary but unconfirmed report (12) has been published which indicates that insulin enhances creatine resorption in heart muscle *in vitro*. Fitch and Shields (13) have shown that the transport of creatine in isolated preparations of the extensor digitorium longus muscle of the rat is an energy dependent, saturable process which can operate against a concentration gradient. Creatine transport in skeletal muscle, therefore, appears to be an active, rather than a passive process of simple diffusion and may well be influenced by hormones such as insulin. On the other hand, it is also possible that the effect of insulin on creatinuria and hypercreatinemia is an indirect one, mediated perhaps by other hormones. It has been reported recently that the administration of growth hormone promotes the accumulation of labeled creatine in skeletal muscle of rats injected with creatine-^{14}C (14). In this connection, the synergistic effect of growth hormone and insulin on amino acid and protein metabolism have long been recognized (15). Whether or not a relationship exists between insulin and growth hormone so far as creatine transport and metabolism is concerned is not known. An alternative explanation for the suppression of hypercreatinemia by insulin could be related to an effect of the hormone on the liver. Presumably, a large fraction of creatine administered ip ultimately passes into the portal circulation before entering the systemic circulation. It is possible that the liver could limit the amount of exogenous creatine entering the systemic circulation by increasing the storage or retention of creatine under the influence of insulin thereby suppressing hypercreatinemia and the associated creatinuria.

Creatinuria is associated with a variety of conditions having no obvious etiologic relationship with one another (16). To what extent, if any insulin can suppress or prevent creatinuria and hypercreatinemia known to be associated with such conditions, *e.g.*, starvation, tissue atrophy, primary diseases of muscle, and so forth, remains to be established.

Summary. The effect of insulin on the creatine content of blood and urine was studied in rats which were made creatinuric by the ip injection of creatine. The results indicate that 0.5–2.0 units of NPH insulin administered sc 2 hr prior to the injection of 5 mg of creatine prevented creatinuria induced by creatine loading. When rats were injected with 5 mg of creatine, the blood creatine concentration rose to 190% of the preinjection control value after 60 min and then fell to 180% of the control after 2 hr. The blood creatine concentration in rats treated with 2 units of insulin increased to 114% of the control value 1 hr after creatine loading and then decreased slightly after 2 hr. These results show that the hypercreatinemia which occurs in rats shortly after creatine loading can also be suppressed by insulin, suggesting that the action of the hormone in preventing load-induced creatinuria is related to its effect on the blood creatine concentration, rather than to an effect on the kidneys *per se*.

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