Effect of Insulin on the Uptake of Creatine-1-^{14}C by Skeletal Muscle in Normal and X-Irradiated Rats[1] (36344)

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Animals exposed to X-irradiation exhibit creatinuria (1, 2) which is dose dependent (3) and is maximal between days 2 and 4 after a single whole-body exposure. In a series of studies designed to elucidate mechanism, Gerber *et al.* (4–6) have provided evidence to support the view that radiation-induced creatinuria results in part from a defect in the ability of irradiated muscle to take up creatine from the blood and in part from an indirect effect of X-irradiation. The role of the endocrine glands in the production of creatinuria after exposure to X-rays has not been completely clarified; however, the possibility that radiation-induced creatinuria is mediated by irradiation of the adrenal (7) or the thyroid and parathyroid glands (8) appears to be excluded. Recently, we observed that radiation-induced creatinuria can be prevented in rats by the administration of insulin (9). Although the mechanism by which insulin suppresses creatinuria produced in animals exposed to X-irradiation is not known, several hypotheses have been proposed to explain this novel action of the hormone (9). Other studies have demonstrated that creatinuria induced by creatine loading also can be prevented by treatment with insulin (10). It was also shown in the latter experiments that hypercreatinemia which is observed shortly after the intraperitoneal (ip) injection of a creatine load is suppressed by insulin, suggesting that the action of the hormone in preventing creatinuria is related directly or indirectly to its effect on the blood creatine concentration.

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One way that insulin could prevent radiation-induced, as well as load-induced, hypercreatinemia and the associated creatinuria, is by enhancing or facilitating the transmembrane transport of creatine in skeletal muscle as is the case for the transport of glucose (11) and certain amino acids (12). In the studies described below, we attempted to test this hypothesis by determining if insulin can influence the uptake of creatine-1-^{14}C by skeletal muscle in the normal and X-irradiated rat *in vivo*. In addition, we have studied the effect of insulin on the blood creatine concentration in rats which were exposed to X-rays, since it is known that X-irradiation produces hypercreatinemia, as well as creatinuria (2).

Materials and Methods. Male rats of the Wistar strain (weighing 184 ± 8 g) were fed Purina Lab Chow and given water to drink *ad libitum*. In each experiment, the rats were divided into pairs of equal weight; one member of a pair was treated with X-rays and/or insulin and the other member was treated as a control. Using this experimental design, the following studies were carried out:

Expt. 1. A group of rats was injected subcutaneously (sc) with 2 units of NPH insulin (Squibb Co.) on the morning of the experiment. Twenty-four hours later, the rats were again injected with 2 units of insulin. The dose of insulin used in this experiment was chosen on the basis that 2 units of NPH insulin administered on two successive days to male Wistar rats completely prevents load-induced as well as radiation-induced creatinuria (9, 10). The "paired" controls were treated similarly except that they were inject-

ed sc with 0.2 ml of saline instead of insulin. Two hours after the last injection, all of the animals were injected ip with 5.0 μCi of creatine-1-^{14}C (0.02 mCi/mg). Exactly 1 hr after injection of the isotope, each animal was killed by decapitation and blood was collected in beakers containing a few drops of heparin as an anticoagulant. Whole blood (0.5 ml) was mixed with 1.5 ml of $Ba(OH)_2$ reagent and 1.5 ml of $ZnSO_4$ reagent and deproteinized according to the method of Van Pilsum *et al.* (13). Radioactivity of the protein-free supernatant solution was determined in a Beckman liquid scintillation spectrometer, Model LS-250. The deproteinized samples of blood were assayed for creatine by the α-naphthol micromethod essentially as described by Gerber *et al.* (14).

Immediately after decapitation of the rats, blood was collected, and the skin, connective tissue, and fat were removed from the carcass. The skeletal muscles of the upper part of the hind limbs, the sacrospinalis muscles of the back, and the pectoralis major and minor muscles of the chest were isolated and pooled. The muscles were freed of connective tissue, fat, and nerves as far as possible, washed in two changes of physiological saline solution, and blotted with filter paper to remove traces of blood. The pooled muscles were then frozen and kept at $-20°$ until use, usually after 1–3 days. After thawing, the muscles were weighed and homogenized (10%, w/v) with distilled water at 4° in a Sorvall stainless steel homogenizer.

Three milliliters of the aqueous homogenate were placed in a centrifuge tube in a boiling water bath for 4 min. The boiled homogenate was centrifuged in an International Centrifuge Model UV at 2500 rpm for 10 min. The supernatant solution was lyophilized and then redissolved in 1.0 ml of water. The precipitate was discarded. The solution (0.4 ml) was streaked on 5 $\times$ 24 in. Whatman No. 3 MM filter paper. A standard solution containing creatine, guanidinoacetic acid, and arginine was applied as a single spot to one edge of each chromatogram to serve as a marker. Creatine was isolated by descending chromatography in a butyl acetate–acetic acid–ethanol–water system (3:2:1:1, v/v) for approximately 20 hr (4). The papers were dried and a guide strip representing the standard mixture was cut from the main portion of the chromatogram and sprayed with the α-naphthol reagent (14). Creatine was located by comparison with the "marker" and then was eluted with water from the appropriate areas of the chromatogram. The eluates were frozen, lyophilized, and were dissolved in 2.0 ml of distilled water. The creatine content of the solution was determined by the method of Gerber *et al.* (14). Radioactivity was determined by liquid scintillation spectrometry as described previously.

Expt. 2. Rats were irradiated with 500 rads to the entire body with a medical X-ray therapy machine essentially as described in another report (10). This dose of radiation (500 rads) produces a marked creatinuria in rats (9); nevertheless, the dose is well below the LD_{50} so that mortality was expected to be low during the experiment. Paired controls were sham-irradiated under identical conditions immediately after irradiation of the experimental group. Twenty-four hours after irradiation, all rats were injected ip with 5.0 μCi of creatine-1-^{14}C. One hour after the injection of isotope the animals were sacrificed; the blood and muscles were collected and treated as described in the previous experiments.

Expt. 3. A group of rats were irradiated with 500 rads. Immediately after irradiation, the animals were injected sc with 2 units of NPH insulin. Twenty-four hours later the rats were again injected with 2 units of insulin. Paired controls were sham-irradiated and received 0.2 ml of saline sc immediately after irradiation and again after 24 hr. Two hours after the final injection of insulin or saline, the rats were injected ip with 5.0 μCi of creatine-1-^{14}C. One hour after the injection of labeled creatine the rats were sacrificed; the blood and muscles were collected for analysis.

In another experiment, a group of rats was injected sc with 1 unit of NPH insulin or with 0.1 ml of physiological saline solution. Immediately after the injection the tip of the tail was transected and 0.1 ml of blood was

TABLE I. Specific Activity of Creatine in Muscle of Rats Injected with Creatine-1-^{14}C After Total Body Irradiation and/or Treatment with Insulin.

Treatment	Muscle creatine[a]			
	Treated rats	Untreated control rats	Mean of the differences between the specific activities of creatine isolated from muscle of treated and untreated members of a pair	p value
Insulin, 2 units daily	870 ± 55[e] (9)	655 ± 28[b] (9)	+211 ± 68	≤.020
X-Irradiation, 500 rads	915 ± 107 (16)	1230 ± 112[c] (16)	−316 ± 78	≤.005
X-Irradiation, 500 rads + insulin, 2 units daily	752 ± 136 (8)	810 ± 146[d] (8)	−58 ± 68	ns

[a] Specific activities are expressed as counts per minute per micromole per 100 g of body weight.
[b] Injected sc with 0.1 ml of saline 1 day before and then 3 hr before sacrifice.
[c] Sham-irradiated.
[d] Sham-irradiated and then injected sc with 0.1 ml of saline immediately after treatment and again 3 hr before sacrifice.
[e] Mean ± SE; number in parentheses represents number of rats.

collected in a heparinized capillary pipette. Blood was collected at 0.5, 1, 2, and 3 hr after the injection of insulin or saline. Between bleedings, blood flow from the tail was minimized or completely stopped by dipping the tail of the rat in Gelfoam powder (Upjohn Co.) and thus promoting clot formation. The creatine content of the blood was determined as described in previous experiments.

Results. 1. Effect of insulin on the uptake of creatine-1-^{14}C by skeletal muscle in normal and X-irradiated rats. Preliminary studies indicated that there was considerable variability in the data obtained from experiments done on different days when the specific activity of creatine isolated from muscle was studied in otherwise identically treated animals. For this reason, the method of paired observations (15) was adopted to minimize the daily variation observed in the experiments described below.

Table I shows the effect of the administration of insulin on the uptake of creatine-1-^{14}C in muscle of normal and X-irradiated rats. The data indicate that the specific activity of creatine isolated from muscle of insulin-treated animals injected ip with creatine-1-^{14}C and sacrificed after 1 hr was significantly higher than that observed in the untreated, paired controls. In agreement with the studies of Gerber *et al.* (4), the specific activity of muscle creatine obtained from rats exposed to an X-ray dose of 500 rads to the entire body was significantly lower ($p \leq .005$) than that found in sham-irradiated controls. When rats were exposed to 500 rads and then injected with 2 units of insulin immediately after exposure and again after 24 hr, the specific activity of muscle creatine was not significantly different from that of sham-irradiated, paired controls which did not receive insulin.

2. Effect of insulin on the radioactivity in whole blood of rats injected ip with creatine-1-^{14}C. Table II summarizes the data obtained when the effect of insulin administration on the level of radioactivity in blood was studied in normal and X-irradiated rats 1 hr after the ip injection of 5.0 μCi of creatine-1-^{14}C. Occasionally, samples of blood were obtained from animals which contained very little radioactivity when compared to other animals in that group. In every case, the specific activity of creatine isolated from the muscle of those animals which were suspect also was much lower than that found in the rest of the animals in the

TABLE II. Effect of Insulin on the Radioactivity in Blood of Normal and X-Irradiated Rats After Injection of Creatine-1-^{14}C.

Treatment	Radioactivity in blood[a]			
	Treated rats	Untreated control rats	Mean of the differences between the radioactivity in blood of treated and untreated members of a pair	p value
Insulin, 2 units daily	12,000 ± 890[e] (9)	23,100 ± 1860[b] (9)	−11,100 ± 1660	≤.001
X-irradiation, 500 rads	44,900 ± 2000 (16)	26,200 ± 1490[c] (16)	+18,600 ± 2460	≤.001
X-irradiation, 500 rads + insulin, 2 units daily	28,800 ± 1640 (8)	21,700 ± 2260[d] (8)	+7080 ± 3000	ns

[a] Counts per minute per milliliter of whole blood.
[b] Injected subcutaneously with 0.1 ml of saline 1 day before sacrifice and again 3 hr before sacrifice.
[c] Sham-irradiated.
[d] Sham-irradiated and then injected sc with 0.1 ml of saline and again 3 hr before sacrifice.
[e] Mean ± SE; numbers in parentheses represent number of rats.

group. Such animals and their paired controls were excluded from the experiment since these observations suggested that a viscus of the animals had been erroneously injected rather than the peritoneal cavity.

The results show that the level of radioactivity in blood of rats treated with 2 units of insulin and injected ip with 5.0 μCi of creatine-1-^{14}C was about half that of the untreated controls 1 hour after injection of the isotope. When untreated rats were exposed to 500 rads of total-body X-irradiation, the level of radioactivity in the blood of the irradiated rats injected with creatine-1-^{14}C was nearly twice as high as that of the corresponding paired, sham-irradiated animals. On the other hand, when X-irradiated rats were treated with insulin immediately after exposure and again (on the next day) 2 hr before the injection of creatine-1-^{14}C, the radioactivity in the blood of these animals was not significantly different from that observed in sham-irradiated, paired controls.

3. Effect of insulin on the creatine concentration in whole blood of rats. As shown in Table III, the creatine content of blood of rats treated with 2 units of insulin on each of two successive days and then injected with creatine-1-^{14}C was significantly lower than that of paired controls which were not injected with insulin. When rats were exposed to a dose of 500 rads to the entire body, the creatine content of the blood 24 hr after exposure was significantly higher than that of paired, sham-irradiated controls. Furthermore, when X-irradiated rats were treated with 2 units of insulin immediately after irradiation and then again 24 hr after exposure, the creatine content of the blood after the last injection of insulin was not significantly different from that of the paired, sham-irradiated controls which did not receive insulin.

Table IV shows the kinetics of the change in creatine content of blood obtained from the tail in rats after the sc injection of insulin or 0.1 ml of physiological saline. The average creatine concentration in blood prior to the injection of insulin at time zero was 1.68 mg/100 ml of whole blood. The concentration of creatine in the peripheral blood remained constant for at least 1 hr after the injection of insulin. After 2 hr, however, the creatine content of the blood decreased to 84% of the value at time zero and then decreased further to 79% of the control value after 3 hr. In contrast, the creatine concentration in the peripheral blood of control rats injected sc with 0.1 ml of physiological saline solution did not change significantly

TABLE III. Creatine Concentration in the Blood of Rats Injected with Creatine-1-^{14}C After Total-Body Irradiation and/or Treatment with Insulin.

Treatment	Creatine conc in whole blood[a]			
	Treated rats	Untreated control rats	Mean of the differences between the creatine conc in blood of treated and untreated members of a pair	p value
Insulin, 2 units daily	23 ± 3[e] (9)	32 ± 2[b] (9)	−9.3 ± 1.9	≤.005
X-irradiation, 500 rads	32 ± 3 (16)	24 ± 2[c] (16)	+7.8 ± 2.0	≤.005
X-irradiation, 500 rads + insulin, 2 units daily	21 ± 1 (8)	19 ± 1[d] (8)	+1.8 ± 1.4	ns

[a] Micrograms of creatine per milliliter of whole blood.
[b] Injected sc with 0.1 ml of saline 1 day before and then 3 hr before sacrifice.
[c] Sham-irradiated.
[d] Sham-irradiated and then injected sc with 0.1 ml of saline immediately after irradiation and again 3 hr before sacrifice.
[e] Mean ± SE; numbers in parentheses represent number of rats.

after 3 hr from the value at time zero.

Discussion. If radiation-induced creatinuria is caused by an impaired uptake of creatine in irradiated skeletal muscle as suggested by Gerber *et al.* (4–6), then an agent such as insulin which is able to prevent creatinuria caused by whole-body X-irradiation (9) might be expected to enhance the uptake of creatine by muscle. The results of our experiments support the view that insulin promotes the transport of creatine in skeletal muscle in normal, as well as in X-irradiated rats. Thus, the data in Table I indicate that the uptake of creatine-1-^{14}C by skeletal muscle was impaired in rats exposed to whole-body X-irradiation, and that this impairment was relieved by the administration of insulin. Furthermore, the uptake of labeled creatine by skeletal muscle was enhanced in normal rats treated with 2 units of insulin on each of two successive days. Other evidence is also in agreement with the view that insulin promotes the transfer of creatine from the blood to other body compartments such as skeletal muscle. Consistent with the observation that insulin enhances the uptake of labeled creatine by muscle is the finding that the total amount of radioactivity per milliliter of blood was less in the insulin-treated rats than in the untreated controls 1 hr after the ip injection of creatine-1-^{14}C. In view of the findings in skeletal muscle, this observation is interpreted to mean that labeled creatine was removed more rapidly from the blood in rats treated with insulin than in the untreated controls. In contrast, X-irradiated rats which exhibited an impaired uptake of creatine-1-^{14}C by skeletal muscle showed higher levels of radioactivity in blood than did the sham-irradiated controls, indicating that labeled creatine is removed more slowly from the circulation in rats exposed to whole-body X-irradiation. When X-irradiated rats were treated with insulin, the amount of radioactivity observed in the blood after the injection of creatine-1-^{14}C was not significantly different from that in the paired, sham-irradiated controls. These observations suggest that the defect in the removal of labeled creatine from the circulation exhibited by X-irradiated rats is relieved by the administration of insulin. Finally, the data in Table III show that the changes in blood creatine concentration which occurred in normal and X-irradiated rats treated with or without insulin varied in the same direction as the changes that occurred in the amount of radioactivity found in the

TABLE IV. Concentration of Creatine in Blood of Rats Treated with Insulin.[a]

Treatment	Creatine conc (% of control)[d]; after injection of insulin (hr): 0.5	1	2	3
Saline	100 ± 1[b] (10)[c]	99 ± 1 (10)	98 ± 1 (10)	98 ± 2 (10)
Insulin, 1 unit	99 ± 2 (13)	97 ± 3 (13)	84 ± 3 (13)	79 ± 3 (13)

[a] Samples of tail blood were removed prior to and 0.5, 1, 2, and 3 hr after injection of insulin or physiological saline solution.

[b] Values are means ± SE.

[c] Number in parentheses is the number of observations.

[d] The average creatine concentration in the blood at time zero was 1.68 mg/100 ml of blood.

blood. Thus, insulin-treated rats exhibited a significantly lower blood creatine concentration than did the untreated controls. The data also indicate that the hypercreatinemia which is observed in rats after total-body X-irradiation (2) was diminished by treatment with insulin. Furthermore, kinetic studies (Table IV) show that the effect of insulin on the creatine concentration of the blood in normal rats was already noticeable within 1–2 hr after the administration of the hormone.

Several possible complications in the interpretation of the radioactivity studies carried out in the blood and muscle must be mentioned. First, radiochemically pure creatine was not isolated from the blood in these experiments and therefore it is possible that other radioactive species in addition to creatine may have contributed to the radioactivity found in the blood. However, the only known metabolic reaction that creatine is involved in, other than the phosphorylation of creatine to phosphorylcreatine, is its spontaneous (nonenzymatic) conversion to creatinine, a metabolic "dead end." The metabolic conversion of creatine to creatinine is known to occur very slowly, *in vivo*; the biological half-life of creatine in the rat is about 30 days (16) so that significant formation of labeled creatinine from creatine-1-^{14}C would not be expected to occur after 1 hr. Thus, it is likely that radioactivity measured in the blood in these experiments was due only to the presence of creatine-1-^{14}C and not to the presence of any labeled creatinine.

Second, it must be acknowledged that the measurement of specific activity of muscle creatine represents an average value which includes a contribution from creatine found in the extracellular space, as well as from that found in the intracellular space. Consequently, the results of our experiments do not permit one to decide if insulin promotes the uptake of labeled creatine into the intracellular or into the extracellular compartment of muscle (or both). Furthermore, radioactive creatine was administered intraperitoneally to animals which were either exposed to X-irradiation or received a relatively large dose of insulin or both. It should be noted that individual animals treated with insulin and/or X-rays may have exhibited differences in absorption of labeled creatine from the peritoneal cavity into the blood circulation; consequently, this could have contributed to the high day-to-day variation observed in the specific activity of muscle creatine in these experiments.

Although the results of our studies point to a stimulating effect of insulin on creatine transport in skeletal muscle, it is not clear from these experiments whether we are dealing with a primary or secondary effect of the hormone. It is possible that insulin could indirectly influence creatine transport by its action on a target other than muscle; however, a preliminary, but unconfirmed report (17) indicates that insulin enhances creatine transport in isolated heart muscle, *in vitro*. In order to prove whether or not insulin acts *directly* on skeletal muscle to promote the uptake of creatine, a system such as the one described by Fitch and Shields (18) will have to be used to study the effect of insulin on creatine transport in an isolated muscle

(extensor digitorum longus) preparation.

Summary. The uptake of labeled creatine by skeletal muscle was enhanced in rats treated with 2 units of NPH insulin on each of two successive days and then injected ip with creatine-1-^{14}C. While the uptake of labeled creatine by muscle was impaired in rats exposed to an X-ray dose of 500 rads, this impairment was relieved by treatment with insulin after X-irradiation. The level of radioactivity and the creatine concentration in whole blood was lower in insulin-treated rats injected ip with creatine-1-^{14}C than in untreated controls. In contrast, X-irradiated rats injected ip with labeled creatine exhibited higher levels of radioactivity and a higher creatine concentration in blood than did the sham-irradiated controls. When X-irradiated rats were treated with insulin, the amount of radioactivity and the creatine concentration in the blood 1 hr after the injection of labeled creatine was not significantly different from that in the sham-irradiated controls. These observations are consistent with the view that insulin enhances the transfer of creatine from the circulation into skeletal muscle in normal as well as in X-irradiated rats.

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