isolated hepatocytes. Propranolol (a β blocker) at $10^{-5}M$ concentration had a slight effect (5 percent reduction). Yohimbine ($10^{-7}M$ to $10^{-5}M$) and prazosin ($10^{-7}M$ and $10^{-6}M$) were examined for toxic effects on labeled hepatocyte cultures. No significant loss of DNA was observed at any of the concentrations tested. The results in disintegrations per minute per culture (mean ± standard error of the mean) were control, 1004 ± 78; yohimbine ($10^{-7}M$) 1015 ± 206, ($10^{-6}M$) 902 ± 116, and ($10^{-5}M$) 941 ± 31; and prazosin ($10^{-7}M$) 988 ± 72 and ($10^{-6}M$) 901 ± 128. (For yohimbine, $P \geq 0.3$; for prazosin, $P \geq 0.5$).

The effects of lower doses ($10^{-9}M$ and $10^{-8}M$) of prazosin and yohimbine on the dose-response curve for norepinephrine are shown in Fig. 2, a and b. The ability of prazosin to shift the curve to the right is indicative of competitive inhibition for the α_1-adrenergic receptor. We have seen no significant stimulation of thymidine incorporation by clonidine, a selective α_2 agonist, or by isoproterenol, a β agonist. Epinephrine produces a dose-dependent stimulation of DNA synthesis similar to that of norepinephrine, which is also significantly inhibited by prazosin (data not shown). Liver has a high relative concentration of α_1-adrenergic receptors (*6*), and the ability of catecholamines to directly stimulate DNA synthesis through α_1 mediation suggests a significant role for these receptors in the regenerative response.

Our studies do not indicate whether the effect of norepinephrine at α_1 adrenoreceptors involves the stimulation of Ca^{2+} mobilization or phosphatidylinositol turnover. Both of these proceses occur in rat hepatocytes after the addition of norepinephrine (*5, 7*). Earlier studies (*8*) showed that vasopressin and angiotensin II also stimulate DNA synthesis in primary cultures of hepatocytes. These studies and our own indicate that at least part of the regenerative response after partial hepatectomy may be mediated by Ca^{2+} movements or phosphatidylinositol turnover, or both.

To our knowledge, this is the first evidence for regulatory effects of α_1-adrenergic receptors on DNA synthesis. Preliminary data indicate that norepinephrine is present in micromolar concentrations in the plasma of rats after partial hepatectomy, although it remains to be seen whether plasma catecholamines or those delivered by synaptic mechanisms are an important part of the regenerative stimulus. In view of the presence of the α_1-adrenergic receptor subtype in most mammalian tissues, and the potential of this receptor for the stimulation of protein kinase C (phorbol ester receptor) (*9, 10*) via diacylglycerol, the role of the α_1 receptor as a potential regulator of cell growth merits further study.

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Vitamin E Reversal of the Effect of Extracellular Calcium on Chemically Induced Toxicity in Hepatocytes

Abstract. *Isolated rat hepatocytes were incubated in the presence or absence of extracellular calcium and α-tocopherol succinate with three different toxic chemicals; namely, adriamycin in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea, ethyl methanesulfonate, and the calcium ionophore A23187. In the absence of extracellular calcium these three compounds were far more toxic to the cells than in its presence. The addition of vitamin E to calcium-free medium, however, protected hepatocytes against toxic injury, whereas cells incubated in medium containing calcium were not protected. Hepatocyte viability during each toxic insult correlated well with the cellular α-tocopherol content but not with the presence or absence of extracellular calcium. These results suggest that cellular α-tocopherol maintains the viability of the cell during a toxic insult and that the presence or absence of vitamin E in the incubation medium probably explains the conflicting reports on the role of extracellular calcium in toxic cell death.*

It is apparent from the numerous hypotheses that have been proposed to explain the mechanism by which chemicals produce toxic effects in biological systems (*1–3*) that this phenomenon is not well understood. One hypothesis is that chemically induced cell death is the consequence of the influx and accumulation of extracellular calcium ions in the cell (*2*). Results obtained with primary cultures of adult rat hepatocytes showed that toxic manifestations caused by various chemicals were expressed only by cells incubated with Ca^{2+} (*2*). In the absence of extracellular Ca^{2+}, cultured hepatocytes were protected from chemically induced cell death. These findings led to the hypothesis that the influx of

Table 1. α-Tocopherol content of isolated hepatocytes incubated in the presence or absence of extracellular Ca^{2+}, vitamin E, and a toxic chemical. Hepatocyte samples were obtained from the experiments described in the legend to Fig. 1. Rapid separation of viable from nonviable hepatocytes and medium was accomplished by the dibutyl phthalate centrifugation method (*18*). The concentration of α-tocopherol was measured in hexane extracts of viable hepatocytes with the use of an HPLC method (*19*). The average initial cellular α-tocopherol concentration was 0.11 ± 0.01 nmol/10^6 cells. Values represent the means and standard errors of four separate experiments.

Treatment	Cellular α-tocopherol (percentage of initial concentration)			
	Medium minus vitamin E		Medium plus vitamin E	
	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}
Control				
1 hour	74 ± 12	90 ± 7	1229 ± 147	201 ± 16
3 hours	41 ± 9	72 ± 9	2275 ± 365	672 ± 57
ADR-BCNU (4 hours)	<7	63 ± 1	870 ± 58	94 ± 6
EMS (1 hour)	13 ± 3	39 ± 9	632 ± 79	114 ± 5
A23187 (3 hours)	12 ± 4	28 ± 5	2344 ± 215	265 ± 44

extracellular Ca^{2+} across a damaged plasma membrane is the final common pathway for chemically mediated cell death (*2*). The role of extracellular Ca^{2+} in the toxic injury process, however, has been disputed. In experiments with isolated hepatocytes, a number of toxic chemicals were more lethal to cells incubated in the absence of extracellular Ca^{2+} than in its presence (*3, 4*). Furthermore, the total intracellular Ca^{2+} concentration was not increased in hepatocytes exposed to toxic concentrations of these chemicals in the absence or presence of extracellular Ca^{2+} (*4*). These results suggest that chemically induced cell death is not dependent on the influx of extracellular Ca^{2+}.

We considered the possibility that a biochemical difference between the experimental systems used in the studies described above—that is, cultured hepatocytes (*2*) and isolated hepatocyte suspensions (*3, 4*)—may be responsible for the observed discrepancy in the role of extracellular Ca^{2+}. Although freshly isolated hepatocytes placed in culture for 16 to 24 hours undergo numerous biochemical and morphological changes (*5*), studies with cultured hepatocytes showed that the elimination of extracellular Ca^{2+} accelerated $CdCl_2$ toxicity (*6*). Because these results were in agreement with those from earlier studies in which freshly isolated hepatocytes were used, cellular alterations resulting from placing hepatocytes in culture were apparently not responsible for the conflicting reports.

Differences in the composition of the incubation medium were also prevalent in the previously described experimental systems. Williams' medium E had been used exclusively in the studies showing that toxic cell death occurs in the presence of extracellular Ca^{2+} but not in its absence (*2*). In contrast to the media used by investigators who dismissed the need for extracellular Ca^{2+} in toxic cell death (*3–5*), Williams' medium E contains the antioxidant vitamin E (*7*). Vitamin E (α-tocopherol) is an important cellular protective constituent that prevents peroxidative damage in membranes (*8*) and may be a preventive agent for chemically induced carcinogenesis (*9*). We therefore examined the effect of exogenous vitamin E on chemically mediated cell death in the presence or absence of extracellular Ca^{2+}. The results (Fig. 1) show that the addition of vitamin E succinate to the incubation medium protected isolated hepatocytes incubated in Ca^{2+}-free medium against chemically induced cell death. Hence chemically induced cell death may be dependent, not on the presence of extracellular Ca^{2+} but rather on cellular α-tocopherol (Table 1).

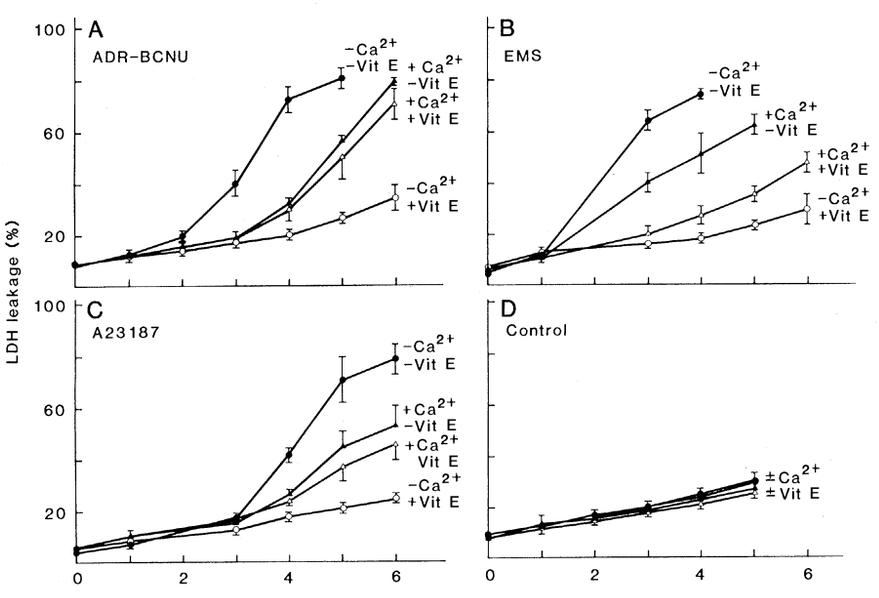

Fig. 1. The effect of extracellular Ca^{2+} on chemically induced cell death is reversed by the addition of vitamin E to the incubation medium. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (175 to 225 g) by the method of collagenase perfusion (*15, 16*). A yield of 5×10^8 to 6×10^8 cells per liver with greater than 90 percent viability (trypan blue exclusion) was obtained. Hepatocyte suspensions were prepared at 3×10^6 cells per milliliter for a total volume of 20 ml in a modified Fisher's medium with or without 3.5 m*M* $CaCl_2$. Fisher's formulation (*17*) was modified as follows: phenol red, methionine, cystine, glutamine, $CaCl_2$, penicillin, streptomycin, and horse serum were omitted; and alanine (50 μg/ml), aspartate (60 μg/ml), hydroxyproline (10 μg/ml), glycine (50 μg/ml), proline (40 μg/ml), and ornithine (20 μg/ml) were added. Immediately before use, the medium was supplemented with 10 m*M* Hepes and 0.2 m*M* cystine and was oxygenated, and the *p*H was adjusted to 7.4. Hepatocyte suspensions were placed in 125-ml culture flasks and slowly rotated at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2. After 15 minutes, zero-time samples were taken, and hepatocyte suspensions in the presence and absence of extracellular Ca^{2+} were incubated with and without α-tocopherol succinate (final concentration, 25 μ*M*). Suspensions were then treated with one of the compounds listed below and monitored hourly for loss of viability. Cell death was determined by measuring the leakage of cellular LDH (lactate dehydrogenase) into the medium (*4, 15*). Leakage of LDH is expressed as the percentage of total cellular LDH. Separate experiments were performed with each of the following compounds: ADR-BCNU [adriamycin (250 μ*M*) in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (115 μ*M*) (supplied by the National Cancer Institute)]; EMS [ethyl methanesulfonate (50 m*M*) (cell concentration, 2×10^6 cell/ml)]; A23187 (5 μ*M*); dimethyl sulfoxide (vehicle control, 10 μl/ml). Two of the toxic compounds were dissolved in dimethyl sulfoxide; EMS was added directly to the suspensions. Values represent the means and standard errors of four separate experiments.

Freshly isolated rat hepatocytes were used to investigate the toxic effects of three different compounds: adriamycin in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); ethyl methanesulfonate (EMS); and the Ca^{2+} ionophore A23187. These toxic chemicals differ in their need for bioactivation to induce cytotoxicity and in their effect on intracellular biochemical processes (*2–4, 10, 11*). Adriamycin, an anthracycline quinone, is a clinically important antitumor agent with cardiotoxic properties. The metabolic activation of adriamycin and subsequent redox cycling lead to the generation of reactive oxygen species that are thought to be the source of adriamycin's cytotoxic effects (*10, 11*). Normally, reactive oxygen species such as hydrogen peroxides are eliminated by glutathione peroxidase with the oxidation of the reduced form of glutathione (GSH) to the oxidized form (GSSG). The subsequent reduction of GSSG by glutathione reductase ensures an adequate supply of GSH. However, in the presence of BCNU, an inactivator of glutathione reductase (*12*), adriamycin treatment results in a 90 percent reduction in cellular GSH levels and in membrane damage, as suggested by a marked increase in lipid peroxidation (*11*). The result of these biochemical alterations is cell death.

The other two toxic compounds, EMS and Ca^{2+} ionophore A23187, have been used in studies of the role of extracellular Ca^{2+} in chemically induced cell death (*2–4*). The cytotoxic potential of EMS, and presumably of A23187, results from the

parent compound. Ethyl methanesulfonate is a mutagen that alkylates numerous tissue macromolecules, including membrane constituents, whereas the ionophore A23187 is a membrane-active compound that facilitates the movement of calcium ions across cellular membranes and down concentration gradients.

The effects of the three liver cell toxins on the viability of isolated hepatocytes incubated in the presence or absence of extracellular Ca^{2+} and in the presence or absence of vitamin E were remarkably similar. Figure 1 shows that in the absence of extracellular vitamin E, the toxic effects of adriamycin-BCNU, EMS, and A23187 (as measured by the increase in extracellular lactate dehydrogenase) were accelerated in hepatocytes incubated without extracellular Ca^{2+}. The viability of hepatocytes exposed to dimethyl sulfoxide (control), however, was not affected by the elimination of either extracellular Ca^{2+} or vitamin E (Fig. 1). This increase in the rate of cell death coincided with an accelerated loss of cellular α-tocopherol (Table 1). In fact, during the toxic insult, the α-tocopherol content of hepatocytes incubated in Ca^{2+}- and vitamin E–free medium was reduced to approximately 10 percent of its initial value before lactate dehydrogenase leakage was observed. The loss of cellular α-tocopherol (Table 1) was also accelerated in control hepatocytes incubated in Ca^{2+}- and vitamin E–free medium.

The effect of extracellular Ca^{2+} on cell death resulting from exposure to adriamycin-BCNU, EMS, or A23187 was reversed by the addition of vitamin E succinate (25 μ*M*) to the incubation medium (Fig. 1). Under these conditions, the rate of toxic cell death was greater in hepatocytes incubated with extracellular Ca^{2+} than in cells incubated in Ca^{2+}-free medium. In fact, hepatocytes incubated in Ca^{2+}-free medium with vitamin E succinate were protected against chemically mediated cell death during the 6-hour incubation period (Fig. 1). Furthermore, loss of intracellular K^+ was no greater in these hepatocytes than in controls (results not shown), suggesting that Ca^{2+}-free medium containing vitamin E succinate protects hepatocytes from chemically induced cell injury as well as cell death. Analysis of these protected cells for α-tocopherol content showed an increase that was six to ten times that observed for unprotected cells incubated with extracellular Ca^{2+} and vitamin E succinate (Table 1). A similar increase was observed for the α-tocopherol concentration in control hepatocytes. After a 1-hour incubation, the α-tocopherol content of control cells incubated with Ca^{2+}-free medium and vitamin E succinate was approximately 1.1 nmol per 10^6 cells or 1200 percent of the initial value (Table 1). This increase accounted for approximately 12 percent of the total vitamin E succinate added to the medium. In contrast, a 1-hour incubation of control hepatocytes in medium containing Ca^{2+} and vitamin E succinate resulted in only a modest increase in the cellular α-tocopherol concentration from 0.1 to 0.2 nmol per 10^6 cells.

We conclude that the presence or absence of vitamin E in the incubation medium explains the conflicting reports on the effects of extracellular Ca^{2+} on hepatocytes exposed to toxic chemicals. It appears that chemically induced liver cell death may be dependent, not on the presence of extracellular Ca^{2+}, but rather on cellular α-tocopherol. Recent results from our laboratory indicate that the α-tocopherol content of cultured hepatocytes, like that of isolated hepatocytes (Table 1), is markedly increased during incubation with Ca^{2+}-free medium and α-tocopherol succinate (*13*). The large increase in the concentration of cellular α-tocopherol appears to be responsible for protecting these hepatocytes from reversible and irreversible cell injury (Table 1). It is unclear, however, whether the protection afforded hepatocytes results from the action of cellular α-tocopherol alone or from an interaction with several other cellular protective systems such as GSH and vitamin C (*14*). We have observed in control hepatocytes as well as in cells exposed to toxic concentrations of adriamycin-BCNU that incubation in Ca^{2+}- and vitamin E–free medium accelerates the loss of both cellular α-tocopherol (Table 1) and GSH (*4, 15*). We therefore suggest that the accelerated depletion of these cellular protective systems is responsible for the increased susceptibility of these cells to chemically induced cell death.

The apparent requirement of cellular α-tocopherol, a membrane-bound antioxidant and free radical scavenger, for maintaining hepatocyte viability during a toxic insult is consistent with the hypothesis that cell membrane damage is the critical event in toxic liver cell injury. Since the site of injury is currently unknown, future studies should examine specific cell membranes such as endoplasmic reticulum, mitochondria, and the plasma membrane for α-tocopherol content and functional integrity during a toxic chemical insult. A better understanding of the mechanism of uptake and cellular distribution of α-tocopherol may elucidate the factors that determine whether or not toxic injury is expressed in biological systems.

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18. We have developed a rapid centrifugation technique that separates viable hepatocytes from nonviable cells and medium (*4*, *15*). In the present study, 0.45 ml of dibutyl phthalate (density, 1.046) was layered over 0.55 ml of 36 percent Percoll (density, 1.06) in a microcentrifuge tube. Rapid separation of cells and medium was accomplished by layering 0.75 ml of isolated hepatocyte suspension over the dibutyl phthalate oil layer and centrifuging for 15 seconds at 13,000*g*. As a result, viable hepatocytes (density, 1.07) were forced through the oil layer and pelleted under the Percoll layer. In contrast, nonviable cells (density 1.02) and medium remain above the oil layer after centrifugation.
19. The measurement of α-tocopherol levels were obtained by adding the internal standard, γ-tocopherol (0.6 nmol in 40 μl of ethanol) to samples of viable hepatocytes. The samples were diluted with 50 percent ethanol (0.4 ml), sonicated, and extracted twice with 0.5 ml of hexane (*20*). Next, the hexane extract was evaporated to dryness with N_2, the residue was dissolved in 0.6 ml of methanol, and 200 μl was analyzed by high-performance liquid chromatography (HPLC). Measurements of α-tocopherol were made on a Spectra-Physics 8700 chromatograph equipped with a Schoeffel 970 fluorescence detector (excitation filter, 205 nm; emission filter, 300 nm (*20*), and a Spherisorb ODS column (20 cm by 4.6 mm; 5-μm particles; Custom LC Inc., Houston). The mobile phase, 70 percent A (80 percent methanol), 30 percent B (100 percent methanol) was run isocratically for 5 minutes and then for 15 minutes at a linear gradient to 1 percent A and 99 percent B at a flow rate of 1.5 ml per minute. The HPLC retention time for γ- and α-tocopherol was approximately 20 and 21 minutes, respectively. Intracellular α-tocopherol concentrations were corrected for the number of viable hepatocytes sampled as determined by DNA content (*4*, *15*) and expressed per 10^6 cells. The lower limit of detection for α-tocopherol was approximately 10 pmol per 10^6 cells.
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Evidence for a Detrimental Effect of Bicarbonate Therapy in Hypoxic Lactic Acidosis

Abstract. *Lactic acidosis, a clinical syndrome caused by the accumulation of lactic acid, is characterized by lactate concentration in blood greater than 5 m*M. *Therapy usually consists of intravenous sodium bicarbonate ($NaHCO_3$), but resultant mortality is greater than 60 percent. The metabolic and systemic effects of $NaHCO_3$ therapy of hypoxic lactic acidosis in dogs were studied and compared to the effects of sodium chloride or no therapy. Sodium bicarbonate elevated blood lactate concentrations to a greater extent than did either sodium chloride or no treatment. Despite the infusion of $NaHCO_3$, both arterial* pH *and bicarbonate concentration decreased by a similar amount in all three groups of dogs. Additional detrimental effects of $NaHCO_3$ were observed on the cardiovascular system, including decreases in cardiac output and blood pressure that were not observed with either sodium chloride or no treatment. Thus there is evidence for a harmful effect of $NaHCO_3$ in the treatment of hypoxic lactic acidosis.*

The administration of sodium bicarbonate ($NaHCO_3$) to patients with metabolic acidosis has become a mainstay of therapy despite limited demonstration of its efficacy in many clinical situations. In cases where metabolic acidosis is largely secondary to actual loss of bicarbonate from the body (such as in renal tubular acidosis), long-term administration of $NaHCO_3$ has been successful (*1*). However, where acidosis is secondary to increased generation of organic acids (in diabetic ketoacidosis and lactic acidosis), the success is less clear (*2*). Both clinical results and those from laboratory studies suggest that bicarbonate may be of no benefit or may actually be harmful under such circumstances (*3–9*). Even in vitro, addition of $NaHCO_3$ to acidotic blood results in a decline of the *p*H (*10*).

Earlier studies from our laboratory have shown that, in three different animal models of lactic acidosis, therapy with $NaHCO_3$ leads to decreased cardiac output, a decline in blood pressure, increased concentrations of lactate in blood, decreased lactate metabolism, and increased subject mortality (*11*, *12*). These studies all dealt with type B (no clinical evidence of hypoxemia) lactic acidosis. A more common clinical problem is type A (hypoxic) lactic acidosis, which can occur with hypoxemia or shock states of various origin, resulting in a patient mortality rate of more than 50 percent when blood lactate concentrations exceed 5 m*M* (*13*). We have developed an animal model of hypoxic lactic acidosis (*14*) that facilitates a controlled study of the metabolic and systemic effects of $NaHCO_3$ in the treatment of this disorder.

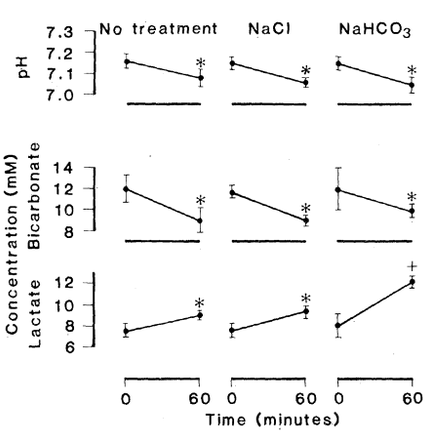

Fig. 1. Blood *p*H and bicarbonate and lactate concentrations in dogs (values are means ± standard error of the mean; $n = 7$ per group) during 60 minutes of therapy of hypoxic lactic acidosis. (*) $P < 0.05$ compared to control at 0 minutes. (+) $P < 0.01$ compared to NaCl as well as animals who received no treatment.

Hypoxic lactic acidosis was induced in intubated anesthetized dogs with controlled ventilation by supplying them, via an anesthesia machine, with a hypoxic gas mixture of approximately 8 percent oxygen and 92 percent nitrogen, producing arterial P_{O_2} values of 25 to 30 mmHg. This results in a stable model of lactic acidosis with blood lactate concentrations above 5 m*M* and bicarbonate concentrations below 15 m*M* (*14*). Three groups of seven dogs each were studied for 60 minutes after the development of hypoxic lactic acidosis: (i) a control group ($n = 7$) receiving no treatment except isotonic fluid infusion equal to estimated fluid losses; (ii) another group ($n = 7$) receiving 1*M* NaCl at a dose of 2.5 meq per kilogram per hour; and (iii) a group ($n = 7$) treated with 1*M* $NaHCO_3$ at the same dose of 2.5 meq per kilogram per hour, which corresponds to that used in clinical settings in the therapy of lactic acidosis (*4*, *6*, *8*). Serial measurement of blood *p*H, P_{CO_2}, P_{O_2}, and concentrations of bicarbonate and lactate were performed as described (*15*). These parameters were monitored in all animals by means of catheters inserted at sites appropriate for measuring production of extrahepatic splanchnic (gut) and skeletal muscle (carcass) lactate and hepatic lactate extraction as described (*15*). Hemodynamic measurements were performed by routine methods (cardiac output was measured by the thermodilution technique, and the mean aortic blood pressure at the femoral artery was measured with a Statham transducer) as described (*11*). For changes within the individual groups, statistical analysis was by the *t*-test for paired data. The statistical analysis for multiple comparisons between the three groups was a one-way analysis of variance with a subsequent